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Elevated cAMP levels reverse *Brucella melitensis*-induced lipid peroxidation and stimulate IL-10 transcription in rats

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Abstract

Brucella species are able to survive and replicate within the phagocytic vacuole of macrophages that induce chronic infection in humans and domestic animals. The activation of oxidative bactericidal activity is one of the defense systems which protect the host from the toxic effects of pathogens. The aim of this study was to evaluate lipid peroxidation, NO production, antioxidative system and inflammation during a period of brucella infection in a rat model; in addition to investigate the role of elevated intracellular cyclic AMP on *Brucella*-induced events. *Brucella* significantly induced lipid peroxidation in plasma, liver and spleen by 3–5-fold at 7 days postinfection. NO concentration was significantly elevated in the liver and spleen while unchanged in plasma. Cyclic AMP elevating agent, rolipram, administration (1 mg/kg/day i.p., 3 days) gradually suppressed lipid peroxidation and NO formation to the basal level in plasma and spleen whilst only a slight decrease was observed in liver. *Brucella* considerably decreased SOD activity in the liver and spleen, with rolipram restoring the enzyme activity in liver and activity in spleen being unchanged. Reverse transcriptase PCR analyses showed that *Brucella melitensis* does not alter TNF-α and IFN-γ transcriptions in liver and spleen. The pathogen did not consistently induce nitric oxide synthase mRNA transcriptions in animals; even in those housed in the same group. IL-10 transcription was induced by rolipram in spleen but not in liver. Our results suggest that activation of the cAMP/PKA pathway suppressed lipid peroxidation and the elevated NO concentrations caused by *B. melitensis*. Moreover, rolipram induced anti-inflammatory cytokine IL-10 transcription and SOD activity, albeit in a tissue dependent manner.

Keywords: *Brucella melitensis*; cAMP; Cytokine; MDA; Oxidative stress; Rolipram; Lipid peroxidation

1. Introduction

Brucellosis remains a public health and agricultural economic problem in most underdeveloped countries. *Brucella* species are facultative intracellular pathogens and cause chronic infectious disease in many animal species as well as humans. The bacteria survive and multiply within the host's reticuloendothelial system (Gross et al., 2004) and in many cases localize in infected macrophages at specific locations within the body, such as spleen, joints, liver, brain, and bones (Ficht, 2003). The disease generally results in abortion in pregnant females and orchitis in male ruminants, with undulant fever, arthritis, endocarditis and osteomyelitis in humans (Ficht, 2003; Young, 1983). Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram negative bacteria such as *Brucella*, and stimulates neutrophils, monocytes, macrophages and other cell types to produce a number of pro- and anti-inflammatory cytokines (Morris and Ryan, 1987).

Bacteria of the genus *Brucella* are resistance to killing in phagocytic cells. Persistence of the pathogen in the reticuloendothelial system is a primary symptom in human

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infection and may continue over several decades (Ficht, 2003). The precise mechanism(s) of the interaction between the host and the pathogen has not been revealed. It has recently been shown that cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway is stimulated by *Brucella suis* infection in macrophage cells via cAMP elevation. This increase is rapid although transient, being maximal within 30 min of pathogen inoculation and declining to basal level at 3 h post infection (Gross et al., 2003). It was suggested that stimulation of this pathway may be required for the establishment of the bacteria within their host. However, whether *in vivo* activation of this pathway affects immunologic defense mechanisms such as antioxidative system, lipid peroxidation, NO synthesis and inflammation is unclear. cAMP is an important second messenger within cells and is degraded by cyclic nucleotide phosphodiesterases (PDE). Elevated cAMP suppresses TNF-α secretion from peripheral monocytes, macrophages and T cells, production of superoxide anions from neutrophils, and IFN-γ production from Th1 cells, but enhances anti-inflammatory cytokine IL-10 transcription from monocytes (Takano et al., 1998; Jimenez et al., 2001; Houslay et al., 2005). Among 11 PDE families, PDE four enzymes are the major type in macrophages and have the highest selectivity and affinity for cAMP (Houslay et al., 2005), and a well characterized effect in inflammatory and immunocompetent cells (Erdogan and Houslay, 1997; Sanz et al., 2005). Therefore, PDE4 inhibitors such as rolipram have the ability to increase intracellular cAMP levels and thereby suppress the production of many proinflammatory cytokines and alter cellular functions.

Nitric oxide plays an important role in several biological processes such as macrophage-mediated cytotoxic activity against a variety of pathogens including bacteria, viruses and parasites (MacMicking et al., 1997). Such antimicrobial effects of NO have been mostly observed in rodents. This molecule is synthesized by various isoforms of nitric oxide synthase (NOS) which convert L-arginine to form L-citrulline and NO. Inducible NOS (iNOS) expression is stimulated by bacterial proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1 as well as by microbial LPS. It has been demonstrated that rolipram has both inhibitory (Zhang et al., 2002) and stimulatory effects (Koschorreck et al., 2003) on NO synthesis in different cell types. The expression and activity of iNOS have received increasing attention in many diseases. However, the effects of rolipram on NO production and iNOS stimulation have not yet been studied in *Brucella* infections.

Superoxide dismutase (SOD), an antioxidant enzyme, activity is widely regarded as essential for aerobic life because oxidative metabolism produces superoxide and hydrogen peroxide as inevitable by-products. Pathogens face the additional challenge of oxidative intermediates produced by neutrophils and macrophages (Harris, 1992). As indicative marker of oxidative damage, the occurrence of lipid peroxidation was assessed in the present study. The elevation in lipid peroxidation is comparative to the relative degree of oxidative stress imposed to the cells. We measured whether the alteration in cellular antioxidant enzyme activities correlated with the change in malondialdehyde (MDA) concentration as an indicator of lipid peroxidation. MDA is such a molecule, being an oxidative end product of unsaturated fatty acids whose synthesis may be triggered by a variety of proinflammatory mediators and pathogens. However, lipid peroxidation and the effect of elevated cAMP level on *Brucella* infection have not yet been evaluated.

The present study was designed to elucidate the occurrence of lipid peroxidation, oxidative stress and inflammation during the early days of brucella infection in a rat model. The effect of elevated intracellular cAMP level on *Brucella melitensis*-induced changes was also studied. In reality, mice and rats are not infected with *Brucella* in nature, since bacteria are slowly eliminated 2–8 weeks after exposure to the pathogen (Hort et al., 2003). However, mice (Hort et al., 2003; Gross et al., 2004) and rats (Yumuk et al., 2003; Baek et al., 2005) have been used for evaluation of the interaction of *Brucella* with host defense mechanisms, though this is obviously species specific.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–300 g ($n = 6$ in groups) were kept under standard conditions at 22 ± 2 °C and 12 h light/dark cycle. Animals received food and water ad libitum. Experimental procedures were approved by Mustafa Kemal University, Veterinary Faculty Ethics Committee for the use and care of laboratory animals.

2.2. Bacteria and inoculation

B. melitensis (16 M strain) was initially kindly supplied by Dr. Ahmet Kalkan, Faculty of Medicine, Firat University, Turkey. This strain was grown for 48 h on Trypticase Soy Agar supplemented with 0.1% (wt/vol) yeast extract (Merck, Germany), harvested in buffered saline solution (PBS), adjusted spectrophotometrically at 600 nm and diluted to 10^6/ml. For the confirmation of infection, initially six rats were infected by intraperitoneal (i.p.) route with 8×10^4 viable *B. melitensis* in 0.5 ml PBS per animal. Then, all rats were sacrificed under anesthesia, and spleen cultures were carried out on the seventh day of inoculation. All six rats were found to be infected. For the experiments, 42 infected rats were randomly divided into seven groups consisting of six rats in each group. In addition, one control group ($n = 6$) was also included.

Rolipram was dissolved in a small volume of dimethylsulfoxide, then diluted with physiological saline just before injection. Rats received 1.0 mg/kg (BW/day) rolipram (Sigma, Germany) once a day for 3 days. Animals were terminated following 24th, 48th and 72nd hour of drug administration. Dose of rolipram was based on studies