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Full Length Research Paper

Anti-inflammatory and antioxidant effects of IMMUNEPOTENT CRP in Lipopolysaccharide (LPS)-stimulated human macrophages

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IMMUNEPOTENT CRP is a mixture of low molecular weight substances, some of which have been shown to be clinically effective for a broad spectrum of diseases, maintaining the immune system and increasing the quality of life of the patients. To determine whether IMMUNEPOTENT CRP modulates the antioxidant and anti-inflammatory effects in LPS-stimulated human macrophages; U-937 cells were differentiated to macrophages with PMA (10 ng/mL), stimulated with LPS (50 ng/mL), and treated with IMMUNEPOTENT CRP (0, 0.5, 1, 3, and 5 U/mL). Thereafter, the antioxidant (Total antioxidant, CAT, GPx, and SOD), inflammatory (NO, TNF-α, COX-2, PGD_2), IκB phosphorylation, and NF-κB DNA binding activities were evaluated by ELISA and colorimetric enzymatic assays. The IMMUNEPOTENT CRP significantly increased the Total antioxidant, CAT, GPx, and SOD activities, and significantly decreased the NO, and TNF-α production, COX-2 and PGD_2 activities, IκB phosphorylation, NF-κB p50 and p65 subunit DNA binding activity in LPS-stimulated human macrophages (*P<0.05). Our results demonstrated that IMMUNEPOTENT CRP plays an important role in modulating the antioxidant and anti-inflammatory effects through IκB/NF-κB pathway in LPS-stimulated human macrophages, suggesting that IMMUNEPOTENT CRP is an effective therapeutic agent in process involved in oxidative cellular damage and clinical inflammatory diseases.

Key words: Dialyzable leukocyte extract, NF-κB, TNF-α.

INTRODUCTION

Acute inflammation is part of defence response, but the chronic inflammation has been found to mediate a wide variety of diseases, including SIRS (systemic inflammatory response syndrome), vascular diseases, cancer, diabetes, arthritis, Alzheimer's disease, pulmonary and autoimmune disease (Aggarwal, 2004; Karima et al., 1999; Nitin and Anthony, 2007; Salminen et al., 2009; Reed et al., 2009). Signaling via IκB is a key pathway involved in inflammation process, due activation of NF-κB transcription factor that induces the production of the major mediators of inflammation during sepsis including TNF-α and ROS (reactive oxygen species). Targeting of these mediators, especially TNF-α, cyclooxygenase-2 (COX-2), prostaglandins and nitric oxide (NO), has been pursued as a mean of reducing pathologic process (Eigler et al., 1997, Mausumee and Nigel, 2002; Sun et al., 2008; Baron, 1993; Uwe, 2008). During SIRS the release of LPS induces an oxidative stress in the cells that is characterized by an exacerbating production of oxidants that can't be resolved by the antioxidant system, producing damage in the cell

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at level of lipids, proteins, carbohydrates and nucleic acids (Okazaki and Matsukawa, 2009). The endogenous antioxidants that kidnap, repair, and prevent the damage caused by free radicals (NO, PGs and COX-2) are catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Hurtado et al., 2005; Macdonald et al., 2003; Blokhina and Kart, 2003; Jetty and Man, 2000; Duduku et al., 2007). The action of antioxidants are an important line of endogenous defense against free radical effects preventing DNA damage (Kaymak et al., 2008; Paulo et al., 2003). Bovine dialyzable leukocyte extract (IMMUNEPOTENT CRP) is a dialysate of a heterogeneous mixture of low-molecular-weight substances released from disintegrated leukocytes of the blood or tissue lymphoid obtained from homogenized bovine spleen. IMMUNEPOTENT CRP is clinically effective and has been shown to be capable of modify the immune response in a broad spectrum of diseases (Franco-Molina et al., 2004; Franco-Molina et al., 2005; Franco-Molina et al., 2006; Franco-Molina et al., 2007). Our group of research has been shown that IMMUNEPOTENT CRP decreased NO production and modulated the production of inflammatory cytokines in LPS-stimulated murine peritoneal macrophages (Franco-Molina et al., 2005), in LPS-stimulated human peripheral blood mononuclear cells (Franco-Molina et al., 2007), and in murine endotoxic shock (Franco-Molina et al., 2004). Results obtained *In vivo* have been demonstrated that IMMUNEPOTENT CRP improved survival in LPS-induced, murine endotoxic shock (Franco-Molina et al., 2004) and human neonatal sepsis (Rodríguez et al., 2000). Chronic degenerative and neoplastic diseases related to inflammation are the dominant medical problems of populations, and development of effective strategies for their prevention or retardation is critical. The combination of immunomodulatory therapies appears to be the future for the treatment in inflammatory diseases (Paulo et al., 2003). The aim of this study is to determine the antioxidant and anti-inflammatory effects of IMMUNEPOTENT CRP in LPS-stimulated human macrophages.

MATERIALS AND METHODS

IMMUNEPOTENT CRP produced by Laboratorio de Inmunología y Virología de la Facultad de Ciencias Biológicas de la UANL (San Nicolás de los Garza, Nuevo León, México). *Limulus amoebocyte* lysate assay (endotoxin detection kit; MP Biomedicals Inc., Sooton, OH, USA). RPMI-1640 medium (GIBCO, Grand Island, NY, USA). Fetal Bovine Serum (GIBCO^{TM}, USA). LPS, PMA (phorbol myristate acetate) and Trypan blue exclusion dye were obtained from Sigma Aldrich (St. Louis, MO, USA). TNF-α human EIA kit, Nitrite/nitrate colorimetric assay kit, PGD_2-MOX enzyme immunoassay kit, COX activity assay Kit, Glutation assay Kit, Catalase assay kit, and SOD assay kit (Cayman Chemical, USA). Total antioxidant colorimetric assay kit (US Biological, Massachussets, USA). IκB activation (CASE TM Cellular Activation of Signaling ELISA, Superarray, USA). NF-κB DNA binding activity according to the protocol of the Trans-Am kit (Active Motif, Carlsbad California, USA).

IMMUNEPOTENT CRP

IMMUNEPOTENT CRP is a mixture of low molecular weight substances (cut-off of 10-12 kDa) released from the dialysis of disintegrated bovine spleens against water. The IMMUNEPOTENT CRP is then lyophilized, assayed for endogenous pyrogens by the *L. amoebocyte* lysate assay and determined to be free of bacterial contamination by culturing IMMUNEPOTENT CRP in different culture media as well as *In vivo* mice inoculation. The IMMUNEPOTENT CRP obtained from $15X10^8$ leukocytes is defined as one unit 1(U). For the assays, IMMUNEPOTENT CRP was dissolved in RPMI-1640 medium FBS-free.

Cell Lines

The U-937 (Histiocytic lymphoma, human) cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and grown in RPMI-1640 containing 10% FBS. U-937 cells were differentiated to macrophages with PMA (10 ng/mL) and incubated at 37°C in a 5% CO_2 atmosphere during 48 h, and used in all the experiments (Anuraag et al., 2008).

Time dose response curve of LPS on NO and TNF-α production

Macrophages were plated at $1X10^6$ cells/well in 3 mL RPMI-1640 supplemented with 10% FBS. Thereafter, were stimulated with LPS (0, 10, 50, 100, 150, and 200 ng/mL) during 5 and 24 h, to induced a pro-inflammatory effect in macrophages. The supernatants were collected for assay of NO and TNF-α production by colorimetric assay and ELISA kits according to the manufacturer´s specifications, respectively.

Anti-inflammatory response of IMMUNEPOTENT CRP

To determine whether IMMUNEPOTENT CRP modulates TNF-α production in LPS-stimulated macrophages, $1X10^6$ cells/well in 3 mL RPMI-1640 were treated with LPS (50 ng/mL), and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0, 0.5, 1, 3, and 5 U/mL); thereafter, were incubated at 37°C in a 5% CO_2 atmosphere for 5 h. The supernatants were collected and stored at -20°C until analysis, the levels of TNF-α production were measured by ELISA as described in the methods.

Determination of oxidants and antioxidants

To determine the effect of IMMUNEPOTENT CRP on the production and activity of oxidants and antioxidants in LPS-stimulated macrophages, $1X10^6$ cells were treated with LPS (50 ng/mL) and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0, 0.5, 1, 3, and 5 U/mL) and incubated at 37°C in a 5% CO_2 atmosphere for 5 h. The levels of production and activity of oxidants and antioxidants were analyzed as follow, respectively. NO production, the supernatants of each treatment were determined using nitrate-nitrite colorimetric assay kit according to manufacturer's instructions, briefly 40 µL of supernatants were mixed with 40 µL of assay buffer, 10 µL of enzyme cofactor and 10 µL of nitrate reductase, and incubated at room temperature for 3 h (conversion of nitrate to nitrite). After 10 min of incubation in Griess reagent at room temperature the absorbance was measured at 560 nm. The activity of PGD_2 was measured as the stable MOX (methoxylamine) derivative PGD_2-MOX after treatment of the samples with methoxylamine hydrochloride; 3.1 pg/mL detection limits using a PGD_2-MOX enzyme immunoassay was evaluated in

the supernatants of each samples. The peroxidase component of COX-1 and 2 were measured with COX assay kit according to manufacturer's instructions, briefly, the activity was determined with arachidonic acid (AA) as a substrate and N, N, N´, N´-tetramethyl-p-phenylenediamine (TMPD) as co-substrate. Equal amounts of protein (20 µg) were incubated at 25°C in a reaction mixture consisting of AA, TMPD, and heme in 0.1 M Tris-HCl (pH 7.5). The absorbance change, due to oxidation of TMPD during the initial 5 min, was measured at 590 nm. The specific enzyme activities were calculated and indicated as nanomoles per minute per milligram for each sample.

The activity of total antioxidant of each treatment was determined using total antioxidant colorimetric assay kit according to manufacturer's instructions, the supernatants were collected to determine total antioxidant activity, the optical density (OD) was measured at 490 nm. GPx activity, was determined using 15 mM cumene hydroperoxide as substrate, absorbance was read at 340 nm for 2.5 min. One unit of activity is defined as the amount of protein that oxidizes 1 µM of NADPH per min and is expressed as milli units per mg protein. CAT activity was determined using whole cell homogenates from each treatment by measuring the exponential decay of 10 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0 monitored at 240 nm according to manufacturer's instructions. SOD activity of cell homogenates from each sample prepared on ice in 50 mM potassium phosphate buffer (pH 7.8, with 1.34 mM diethylenetriaminepentaacetic acid) was determined, this assay is based on the competition between SOD and an indicator molecule NBT for superoxide production from xanthine and xanthine oxidase. One unit of activity was defined as the amount of protein required to inhibit NBT reduction by 50% of maximum for each cell type tested. Incubation for at least 45 min with 5 mM sodium cyanide was used to inhibit Cu, Zn-SOD activity to measure Mn-SOD activity. Cu, Zn-SOD activity was determined by subtracting Mn-SOD activity from total SOD activity of each sample.

IκB phosphorylation assay

Macrophages ($5X10^3$ cells/well) were seeded into 96-well plates and treated with LPS (50 ng/mL) and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0, 0.5, 1, 3, and 5 U/mL) and incubated at 37°C in a 5% CO_2 atmosphere for 5 h. Thereafter, the IκB activation was analyzed by using CASE TM Cellular Activation of Signaling ELISA, Superarray, according to the manufacturer`s instructions. Briefly, cells were fixed to preserve any activation-specific protein modification, such as phosphorylation, two primary antibodies were used, one antibody recognized only the activated (phosphorylated) form of IκB, while another recognized the specific target protein regardless of its activation state. Following incubation with primary and secondary antibodies, the amount of bound antibody in each well was determined. The absorbance readings were normalized to relative cell number as determined by a cell staining solution.

NF-κB DNA binding activity

To determine whether IMMUNEPOTENT CRP modulates the NF-κB DNA binding activity in LPS-stimulated macrophages, $5X10^6$ cells/75 cm^2 flask in 15 mL RPMI-1640 were treated with LPS (50 ng/mL), and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0, 0.5, 1, 3, and 5 U/mL); and incubated at 37°C in a 5% CO_2 atmosphere for 5 h. Thereafter, the cellular pellets were collected and stored at -80°C until used. To prepare nuclear extract, $5X10^6$ cells were washed with ice-cold phosphate buffered saline and incubated on ice on a shaker, with 400 ∝L of cytosolic lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After 15 min, nuclei have been separated by centrifugation at 5,000 rpm for 10 min. The supernatants containing cytosolic extracts have been removed. The pellet, containing nuclei, has been suspended in 50 ∝L of a second lysis buffer. After 30 min of incubation, nuclei have been clarified by high speed centrifugation. Twenty microgram of nuclear extract has been assayed for the DNA binding activity of NF-κB, according to the protocol of the Trans-Am kit (Active Motif, Carlsbad California, USA). Basically, the DNA binding motif of NF-κB (5´-GGGACTTTCC-3´) is coated to a 96-well plate. When nuclear extracts are added to the plate, transcriptionally active nuclear NF-κB binds to DNA, determining the exposure of an epitope, which is recognized by a primary antibody directed against p50 and p65. A HRP-conjugated secondary antibody provides a sensitive colorimetric reaction, which is quantified by spectrophotometry. Absorbance is measured at 450 nm with a reference wavelength of 655 nm. Data shown were media of at least three independent experiments. Previously, the Trans-Am kit showed a good correlation and better sensitivity compared with an electrophoretic mobility shift assay in detecting the DNA binding activity of NF-κB (Renard et al., 2001).

Trypan blue exclusion cell viability assay

Macrophages were plated at $5X10^3$ cells/well in 96 well plates, and incubated at 37°C in a 5% CO_2 atmosphere overnight. Thereafter, were treated with LPS (50 ng/mL), and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0, 0.5, 1, 3, and 5 U/mL); and incubated at 37°C in a 5% CO_2 atmosphere for 5 and 24 h. Thereafter, the relative percentage of cell viability was determined by trypan blue exclusion.

Statistical analysis

All experiments were performed in triplicate, and statistical analysis of the data was performed by analysis of variances (ANOVA) followed by Dunnett´s and Tukey´s tests. Values of $*P < 0.05$ were considered statistically significant.

RESULTS

NO and TNF-α production

All the LPS treatments increased the NO and TNF-α production compared with the control (0 ng/mL). However; the LPS dose of 50 ng/mL for 5 h of incubation induced the highest production of NO (137 µM/$1x10^6$ cells) and TNF-α (412 pg/$1x10^6$ cells) in macrophages ($*P<0.05$) (Figures 1 and 2).

IMMUNEPOTENT CRP modulates the TNF-α production in LPS-stimulated macrophages

Macrophages shown a basal TNF-α production (186 pg/mL), but LPS stimulation significantly increased the TNF-α production (399.7 pg/mL) ($*P<0.05$). The IMMUNEPOTENT CRP treatments significantly decreased the TNF-α production at doses of 0.5 U/mL (330.4 pg/mL), 1 U/mL (189.7 pg/mL), 3 U/mL (22.1 pg/mL), and 5 U/mL (16.7 pg/mL) in LPS-stimulated

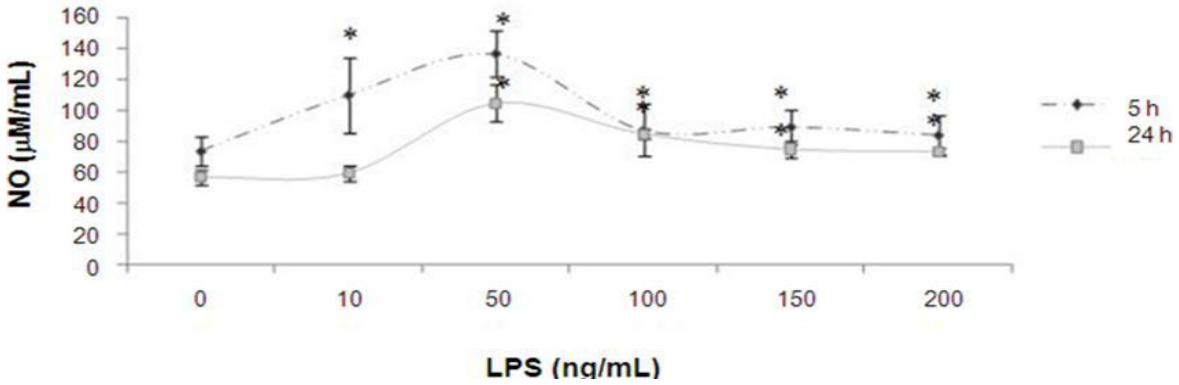

Figure 1. LPS increased the NO production in macrophages. Macrophages (1X10^6 cells/well) were treated with LPS (0, 10, 50, 100, 150, and 200 ng/mL), and incubated at 37°C in a 5% CO_2 atmosphere for 5 and 24 h. The supernatants were collected for assay of NO production at 540 nm (as described in the methods). Determinations were performed on triplicate samples and plotted ± SD (*P<0.05). Results shown are averaged data from three independent experiments.

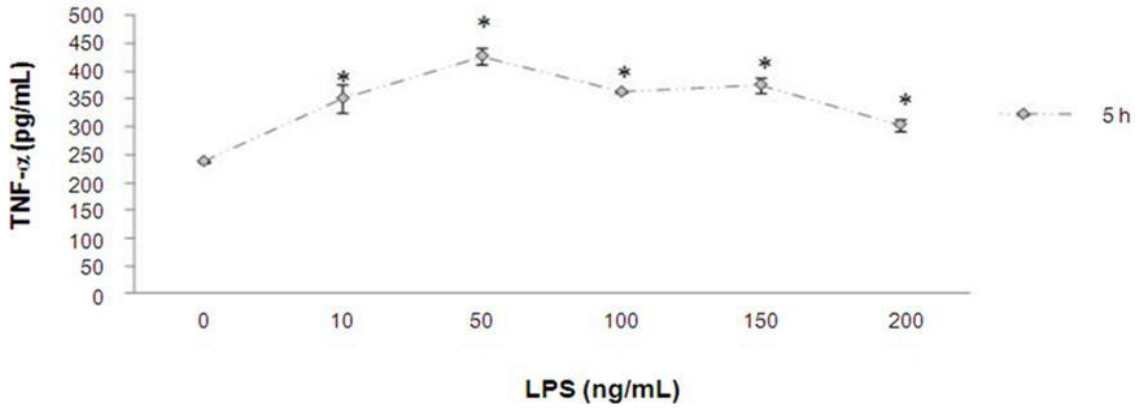

Figure 2. TNF-α production in macrophages stimulated with LPS. Macrophages (1X10^6 cells/well) were treated with LPS (0, 10, 50, 100, 150, and 200 ng/mL), and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. The concentration of TNF-α in supernatants was determined by ELISA (as described in the methods). Determinations were performed on triplicate samples and plotted ± SD (*P<0.05). Results shown are averaged data from three independent experiments.

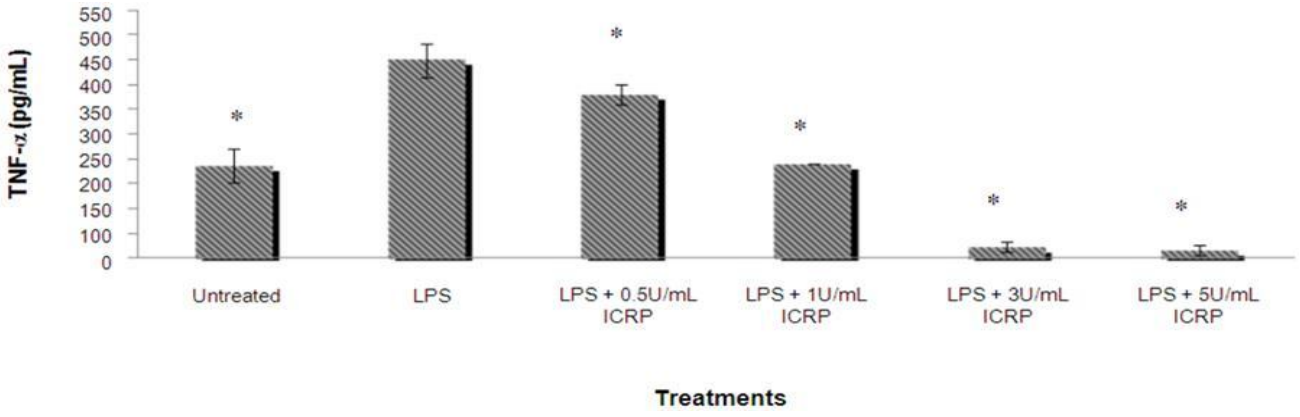

Figure 3. IMMUNEPOTENT CRP decreased the TNF-α production. Macrophages (1X10^6 cells/well) were stimulated with LPS (50 ng/mL), treated with IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. The supernatants of TNF-α production (pg/mL) were evaluated by ELISA (as described in the methods). Determinations were performed on triplicate samples and plotted ± SD (*P<0.05). Results shown are averaged data from three independent experiments. (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

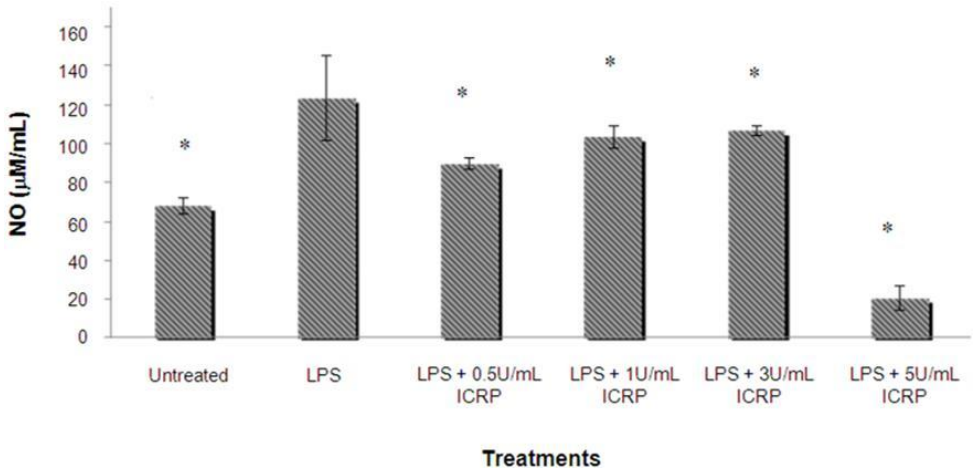

Figure 4. IMMUNEPOTENT CRP decreased NO production in LPS-stimulated macrophages. Macrophages (1X10^6 cells/well) were LPS (50 ng/mL) stimulated, treated with IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. The supernatants were collected for assay of nitric oxide production by nitrite/nitrate colorimetric assay with Greiss reagent. Triplicate samples of each treatment were averaged and plotted ± SD. Results shown are averaged data from three independent experiments (*P<0.05). (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

production at doses of 0.5 U/mL (89.98 µM/mL), 1 U/mL (103.97 µM/mL), 3 U/mL (107.03 µM/mL), and 5 U/mL (20.84 µM/mL) in LPS-stimulated macrophages (*P<0.05) (Figure 4).

IMMUNEPOTENT CRP modulates oxidants and antioxidants

LPS stimulation significantly increased the oxidant and decreased the antioxidant activity (*P<0.05) in macrophages (Figures 5A and B; Figures 6A, B, C, and D, respectively). IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) decreased the oxidant activity of COX-2 [0.5 U/mL (8.59 U), 1 U/mL (7.81 U), 3 U/mL (8.63 U), and 5 U/mL (7.07 U)] (*P<0.05) (Figure 5A) and PGD_2 [0.5 U/mL (17.9 pg), 1 U/mL (14.52 pg), 3 U/mL (10.34 pg), and 5 U/mL (5.9 pg)] activities in LPS-stimulated macrophages (*P<0.05) (Figure 5B) without affected COX-1 activity (*P<0.05) (Figure 5A). When evaluated the antioxidant capacity, IMMUNEPOTENT CRP increased the total antioxidant activity [0.5 U/mL (0.46 mM), 1 U/mL (1.53 mM), 3 U/mL (1.97 mM), and 5 U/mL (2.33 mM)] (*P<0.05) (Figure 6A), GPx [0.5 U/mL (0.71 mU), 1 U/mL (0.86 mU), 3 U/mL (1.47 mU), and 5 U/mL (2.43 mU)] (*P<0.05) (Figure 6B), CAT [0.5 U/mL (2.29 nmol), 1 U/mL (2.75 nmol), 3 U/mL (2.90 nmol), and 5 U/mL (2.70 nmol)] (*P<0.05) (Figure 6C) and SOD [0.5 U/mL (62.21 U), 1 U/mL (58.03 U), 3 U/mL (57.05 U), and 5 U/mL (85.06 U)] (*P<0.05) (Figure 6D) activities in LPS-stimulated macrophages.

IκB phosphorylation

IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) significantly decreased the IκB phosphorylation in LPS-stimulated macrophages [0.5 U/mL (1.40 O.D), 1 U/mL (1.09 O.D), 3 U/mL(1.32 O.D), and 5 U/mL (1.29 O.D) (*P<0.05). LPS stimulation increased the IκB phosphorylation (3.02 O.D) in macrophages (Figure 7).

NF-κB DNA binding activity

NF-κB is one of the main transcription factors involved in the LPS-induced production of TNF-α and others mediators of inflammation. We determined the effects of IMMUNEPOTENT CRP treatment on NF-κB DNA binding activity in LPS-stimulated macrophages. Untreated macrophages shown a basal NF-κB p50 and p65 subunit DNA binding activity. LPS-stimulated macrophages shown an increased NF-κB DNA binding activity (*P<0.05). The IMMUNEPOTENT CRP at doses of 0.5 U/mL did not affect the NF-κB p50 and p65 subunit DNA binding activity, however; doses of 1, 3, and 5 U/mL decreased the NF-κB DNA binding activity in LPS-stimulated macrophages (*P<0.05) (Figure 8).

Effects on cell viability

In all LPS and IMMUNEPOTENT CRP test samples, no significant cytotoxicity was observed (Figure 9).

DISCUSSION

IMMUNEPOTENT CRP decreased the NO and TNF-α production, increased antioxidant molecules and decreased IκB phosphorylation and NF-κB DNA binding activity of p50 and p65 proteins in LPS-stimulated human macrophages. The decreased of NO and TNF-α production were similar to previous results in LPS-stimulated murine peritoneal macrophages (Franco-Molina et al., 2005) and LPS- stimulated peripheral blood mononuclear cells (Franco-Molina et al., 2007). The characteristic of IMMUNEPOTENT CRP to modulate inflammatory and oxidant molecules is very important because these metabolites are potent pro-inflammatory mediators that play an important role in modulating a number of patho-physiological conditions, including cancer, inflammatory and allergic immune response and are considered as potential therapeutic targets (Reed et al., 2009;

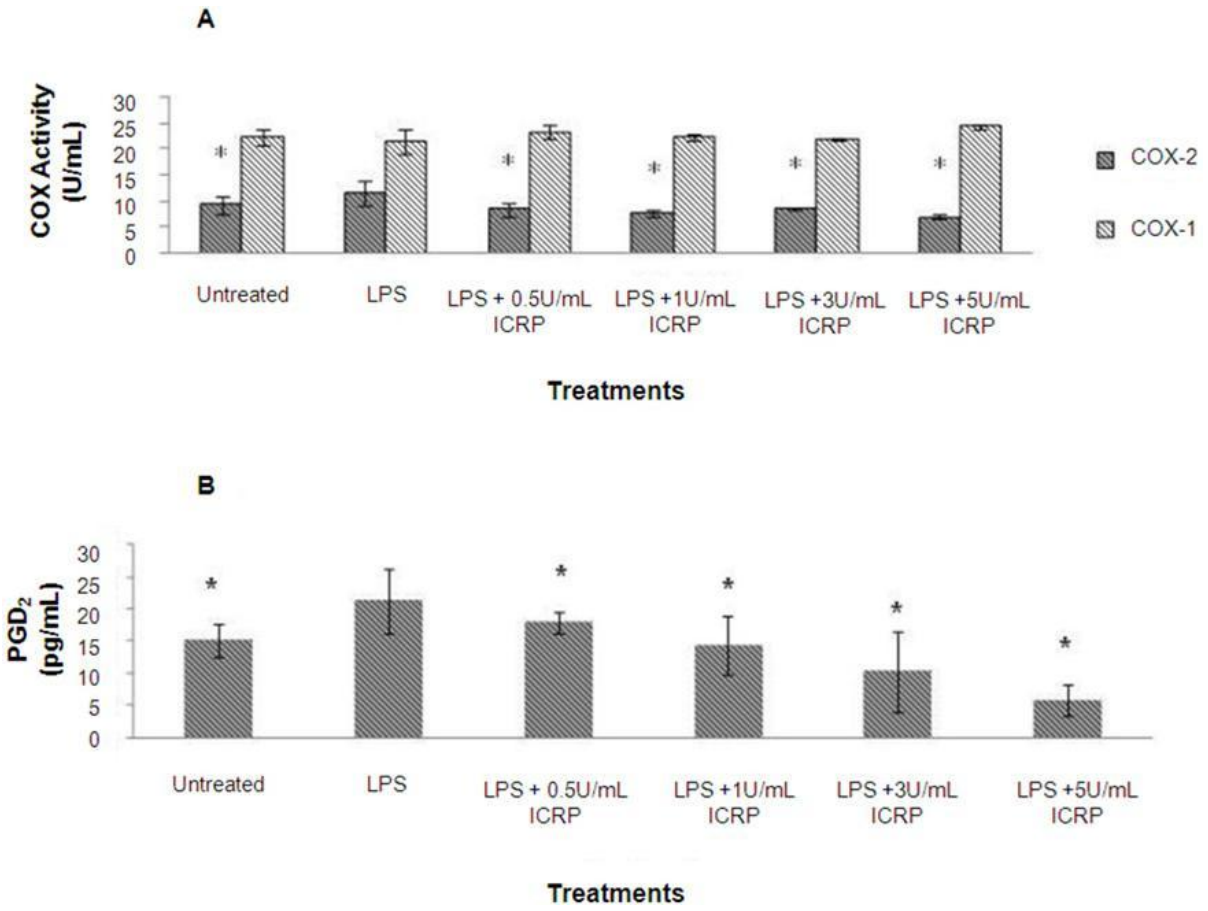

Figure 5. IMMUNEPOTENT CRP decreased oxidants. Macrophages ($1X10^6$ cells/well) were stimulated with LPS (50 ng/mL), treated with IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. (A) The cells were lysated for sonication and evaluated to determine the COX-1 and COX-2 activities as described in the methods. (B) The cells were lysated for sonication and evaluated to determine the PGD_2 production (as described in the methods). Determinations were performed on triplicate samples and plotted ± SD (*P<0.05). Results shown are averaged data from three independent experiments. (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

Eigler et al., 1997; Mausumee et al., 2002). We found similar results (Tanamoto, 1995; Diks et al., 2000; Heumann and Glauser, 1998; Tapping et al., 1999), in where the stimuli of macrophages with LPS cause a released of mediators of inflammation and oxidants substances; however, the treatment with IMMUNEPOTENT CRP decreased all these substances increasing the intracellular antioxidant activity of CAT, GPx, SOD at mitochondrial level, avoiding possible damage collateral induced by oxidative response such as DNA damage, lipid peroxidation and protein structural changes (Jetty and Man, 2000; Duduku et al., 2007; Kaymak et al., 2008; Lee et al., 2010; Park et al., 2010). The IκB phosphorylation and NF-κB DNA binding activities were studied, demonstrating that IMMUNEPOTENT CRP decreased the activity of these molecules. These results open a potential field of study for understanding immunomodulatory action of IMMUNEPOTENT CRP through IκB/NF-κB pathway; because IκB phosphorylation are related with activation of NF-κB transcription factor responsible of release mediators of inflammation (Weinstein et al., 1992).

In summary, can be reasonably speculated that anti-inflammatory and antioxidant activities of IMMUNEPOTENT CRP are mediated by its capacity to modulate in LPS-stimulated macrophages the inflammatory and oxidant metabolites possibly, through IκB/NF-κB pathway (Figure 10) although more studies upstream signalling are needed to clarify the molecular mechanisms of IMMUNEPOTENT CRP to induced its anti-inflammatory and antioxidant effects.

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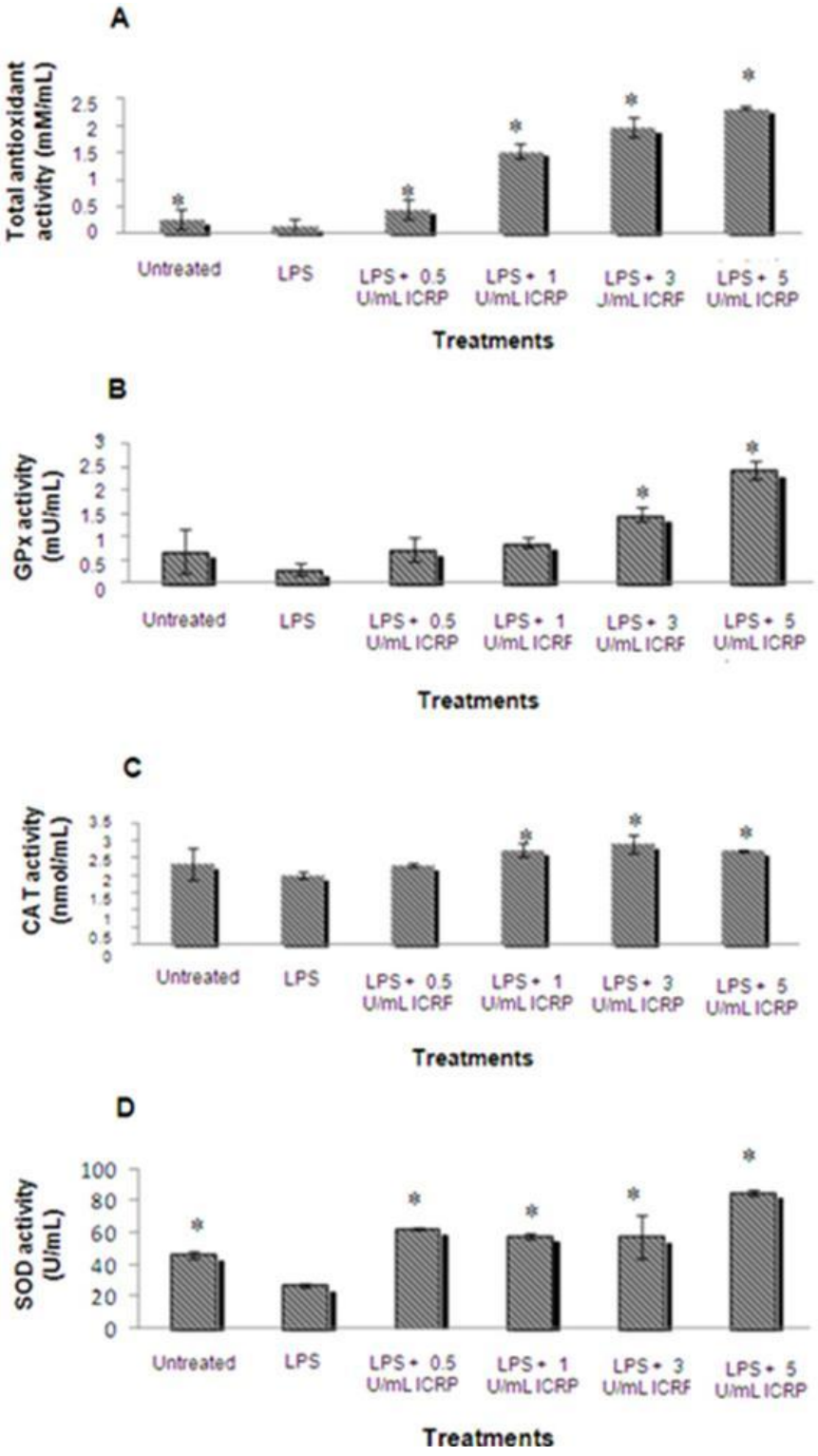

Figure 6. IMMUNEPOTENT CRP increased antioxidant intracellular activity. Macrophages ($1X10^6$ cells/well) were stimulated with LPS (50 ng/mL), treated with IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. The cells were lysated for disruption of cellular membrane by sonication and analyzed the intracellular antioxidant system activity (as described in the methods). **A)** Total antioxidant activity, **B)** GPx activity enzyme, **C)** CAT activity enzyme, **D)** SOD activity enzyme. Determinations were performed on triplicate samples and plotted ± SD. Results shown are averaged data from three independent experiments (*P<0.05). (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

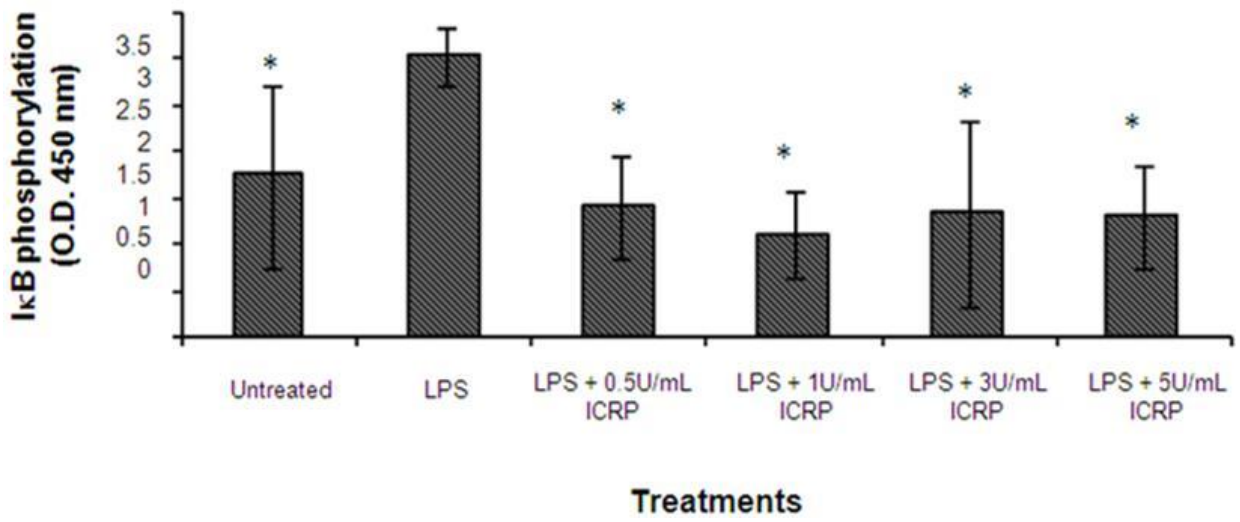

Figure 7. IMMUNEPOTENT CRP decreased IKB phosphorylation in LPS-stimulated macrophages. Macrophages ($5X10^3$ cells/well) were seeded into 96-well plates and treated with LPS (50 ng/mL) and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. Thereafter, the IκB activation was analyzed (by using CASE TM Cellular Activation of Signaling ELISA, Superarray, USA) according to the manufacturer`s instructions. Determinations were performed on triplicate samples and plotted ± SD. Results shown are averaged data from three independent experiments (*P<0.05). (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

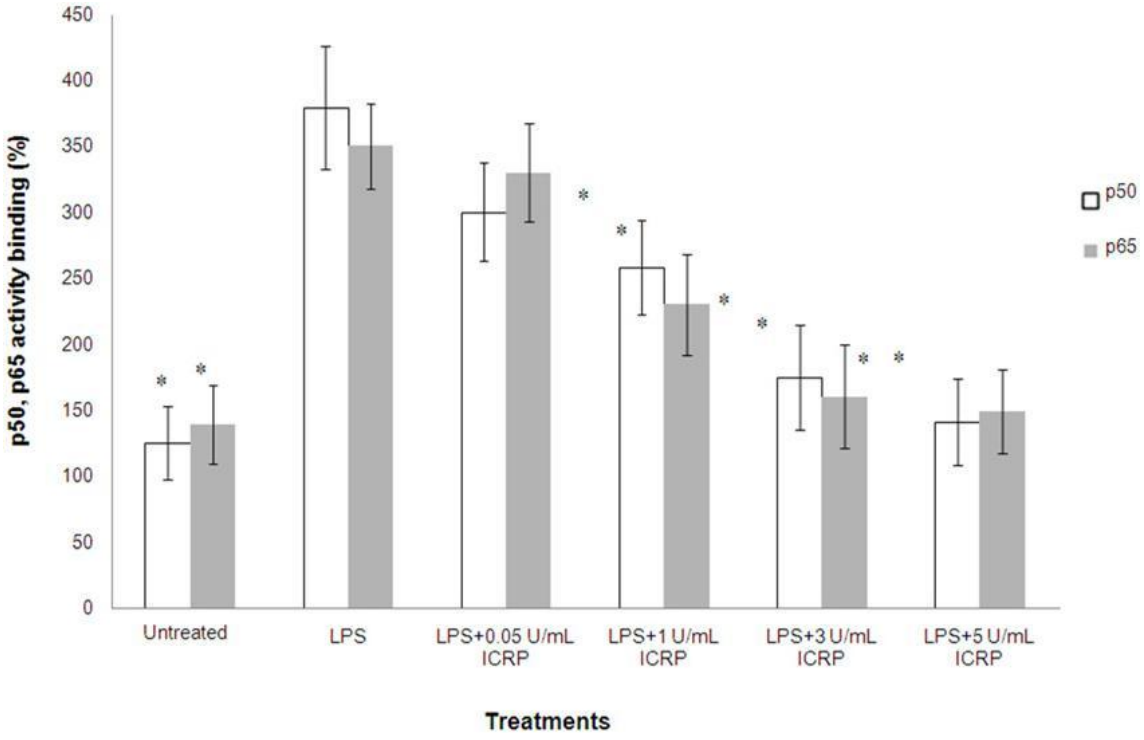

Figure 8. IMMUNEPOTENT CRP affected NF-κB DNA binding activity. Macrophages ($5X10^6$ cells/75 cm^2 flask in 15 mL RPMI-1640) were treated with LPS (50 ng/mL), and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL); and incubated at 37ºC in a 5% CO_2 atmosphere for 5 h. NF-κB activity was determined by measuring binding of the NF-κB subunit p50 and p65 to its NF-κB consensus binding sequence with an ELISA kit. Determinations were performed on triplicate samples and expressed as the percentage of values and plotted ± SD. Results shown are averaged data from three independent experiments (*P<0.05). (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

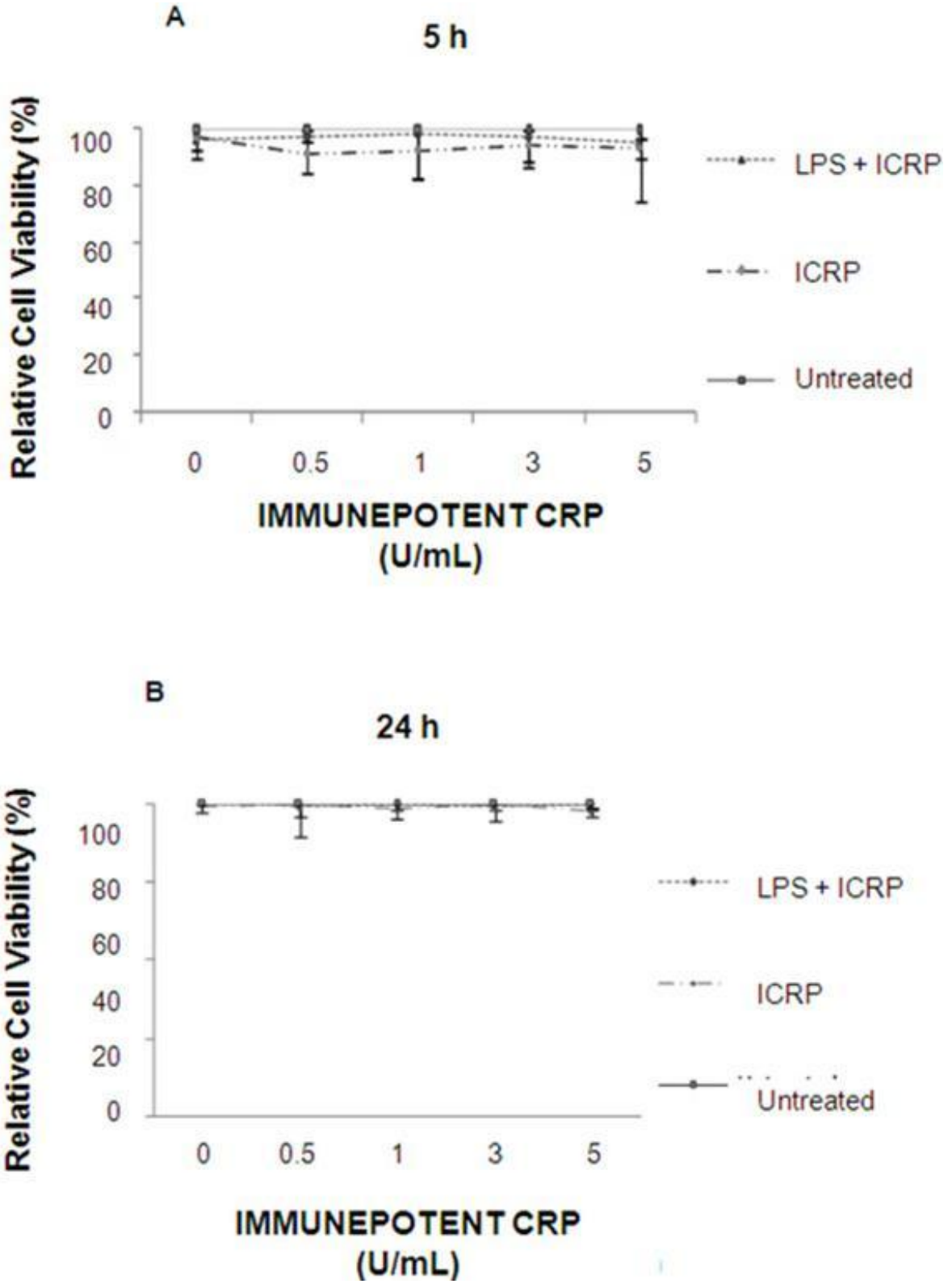

Figure 9. Viability of Macrophages. Macrophages ($5X10^3$ cells/well) were seeded into 96-well plates and treated with LPS (50 ng/mL) and the combination of LPS (50 ng/mL) + IMMUNEPOTENT CRP (0.5, 1, 3, and 5 U/mL) and incubated at 37ºC in a 5% CO_2 atmosphere for 5 and 24 h. Thereafter, the relative percentage of cell viability was determined by trypan blue exclusion. Mean values of three independent experiments ± SD (*P<0.05) were compared with untreated macrophages. (A) Relative cell viability at 5 h. (**B)** Relative cell viability at 24 h. (ICRP = IMMUNEPOTENT CRP, Untreated= macrophages).

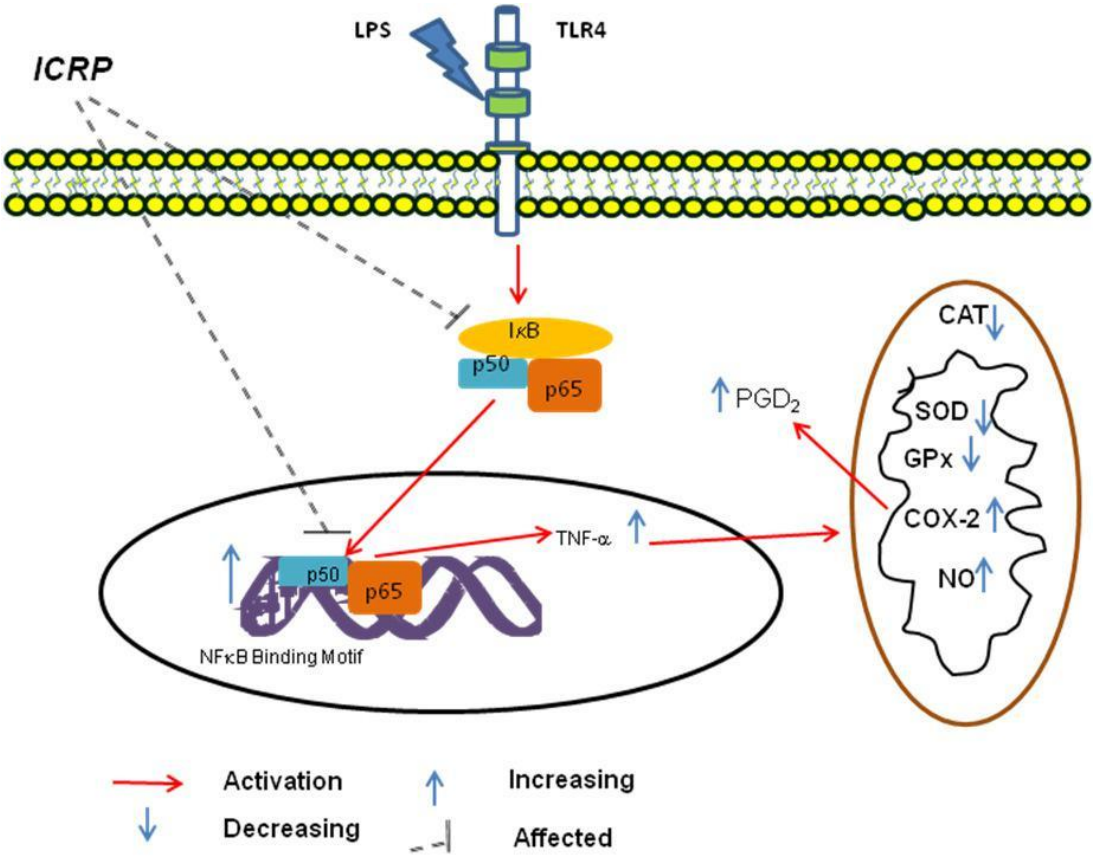

Figure 10. Hypothetical Model of IMMUNEPOTENT CRP Biological Action. The IMMUNEPOTENT CRP significantly increased the Total antioxidant, CAT, GPx, and SOD activities, and significantly decreased the NO, and TNF-α production, COX-2 and PGD_2 activities, IκB phosphorylation, NF-κB p50 and p65 subunit DNA binding activity in LPS-stimulated human macrophages (*P>0.05).

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