ELSEVIER

European Journal of Pharmacology 450 (2002) 85–89

www.elsevier.com/locate/ejphar

Short communication

Activation of RNA-dependent protein kinase and nuclear factor-kB by regulatory RNA from lipopolysaccharide-stimulated macrophages: implications for cytokine production

Fernando L. De Lucca *, Sergio V. Serrano, Liliana R. Souza, Maria Angelica E. Watanabe[1]

Department of Biochemistry and Immunology, School of Medicine University of São Paulo 14049-900, Ribeirão Preto, S.P., Brazil

Received 2 May 2002; received in revised form 2 July 2002; accepted 5 July 2002

Abstract

Our previous results showed that L-RNA, extracted from lipopolysaccharide-stimulated macrophages, induces interleukin-1, interleukin-8 and tumor necrosis factor-α (TNF-α) in resident macrophages. It was demonstrated the promoter of these cytokine genes contain nuclear factor-kB (NF-κB) binding sites. We hypothesized that this effect of L-RNA is mediated by RNA-dependent protein kinase (PKR) through NF-κB activation. We found that L-RNA activates PKR and induces NF-κB activation through degradation of its inhibitor I-κBα. These data support the idea that L-RNA acts as a regulatory RNA. A model for the mechanism of action of L-RNA is proposed.

Keywords: RNA, regulatory; PKR (RNA-dependent protein kinase); NF-κB (nuclear factor-kB); Lipopolysaccharide; Macrophage

1. Introduction

Recent studies have demonstrated that non-coding RNAs, named regulatory RNAs, regulate gene expression and exert their actions at the RNA level (Eddy, 2001; Erdmann et al., 2000; Mattick, 2001). We have previously shown that L-RNA, extracted from lipopolysaccharide-stimulated macrophages, induces the release of the TNF-α, interleukin-1 and interleukin-8 by resident macrophages (Ribeiro et al., 1993, 1995). We hypothesized that L-RNA acts as a regulatory RNA and its effects are mediated by RNA-dependent protein kinase (PKR) through the activation of the transcriptional regulator nuclear factor-kB (NF-κB).

PKR is a serine–threonine kinase which is activated by intermolecular autophosphorylation upon binding to RNA (Clemens and Elia, 1997). It has been shown that the PKR mediates antiviral activity of interferon α/β and its regulation by viral RNAs is well established (Clemens and Elia, 1997). Accumulating evidence during recent years has implicated PKR in cell growth, differentiation and apoptosis (Jagus et al., 1999). These findings raise the possibility that PKR is also regulated by cellular RNAs. However, little is known about the PKR regulation by cellular RNA (Li and Petryshyn, 1991; Nussbaum et al., 2002; De Lucca et al., in press).

The activation of PKR leads to the phosphorylation of the I-κBα (Kumar et al., 1994), an inhibitor of NF-κB, and its subsequent degradation which frees the NF-κB to translocate to the nucleus where it regulates gene transcription. Thus, the degradation of I-κBα is considered as a key step in NF-κB activation (Karin and Ben-Neriah, 2000).

It should be emphasized that the promoters of interleukin-1, interleukin-8 and TNF-α genes contain binding sites for NF-κB (Tak and Firestein, 2001) and we have demonstrated that these pro-inflammatory cytokines are induced by treatment of resident macrophages with L-RNA (Ribeiro et al., 1993, 1995).

In this study, the ability of L-RNA to activate PKR and NF-κB was examined. L-RNA was fractionationed by affinity chromatography and the L-RNA fraction responsible for activating PKR was also investigated. A model for the mechanism of action of L-RNA is proposed.

* Corresponding author. Tel.: +55-16-6023259; Fax: +55-16-6336840.
E-mail address: fldlucca@fmrp.usp.br (F.L. De Lucca).
[1] Present address: Departamento de Ciências Patológicas, Universidade Estadual de Londrina, 86051970 Londrina, PR-Brazil.

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)02072-1

2. Materials and methods

2.1. Animals

Male Wistar rats (*Rattus norvegicus*, 180–200 g) maintained in temperature-controlled rooms at 25 °C with free access to food and water were used as the source of peritoneal macrophages. This study received prior institutional approval according to the guidelines of the ethics committee.

2.2. Treatment of macrophages with lipopolysaccharide

Rat peritoneal cavities were stimulated by the injection of 3% thioglycollate solution (10 ml/animal). Four days later, the peritoneal macrophages were harvested in RPMI medium and allowed to adhere to plastic tissue culture dishes for 1 h at 37 °C in an atmosphere of air containing 5% CO_2. The monolayers (2×10^6 macrophages/well) were then washed three times with phosphate buffered saline (PBS), pH 7.4, and incubated for 1 h at 37 °C in RPMI medium containing 10 ng/ml of *Escherichia coli* lipopolysaccharide. The cells were harvested and used for RNA extraction.

2.3. RNA extraction

RNA was extracted from lipopolysaccharide-stimulated (L-RNA) or non-stimulated (N-RNA) macrophages according to Ribeiro et al. (1995). The macrophage pellet was homogenized in 0.05 M Tris–HCl buffer, pH 9.0, containing 0.01 M KCl and centrifuged at 12 000 × *g* for 15 min at 4 °C. The supernatant was as the source of cytoplasmic RNA which was stored under ethanol at −20 °C until use. The integrity of RNA was routinely evaluated by electrophoresis on 1% agarose.

2.4. Treatment of L-RNA with ribonuclease

The L-RNA preparations were subjected to enzymatic treatment using a mixture containing ribonuclease T_1 and bovine pancreatic ribonuclease type 1-A as described by Passos and De Lucca (1991).

2.5. Oligo(dT)-cellulose chromatography

The poly A(+) and the poly A(−) RNA fractions were separated from the bulk of L-RNA by affinity chromatography on an oligo(dT)-cellulose column according to Bertolini and De Lucca (1986).

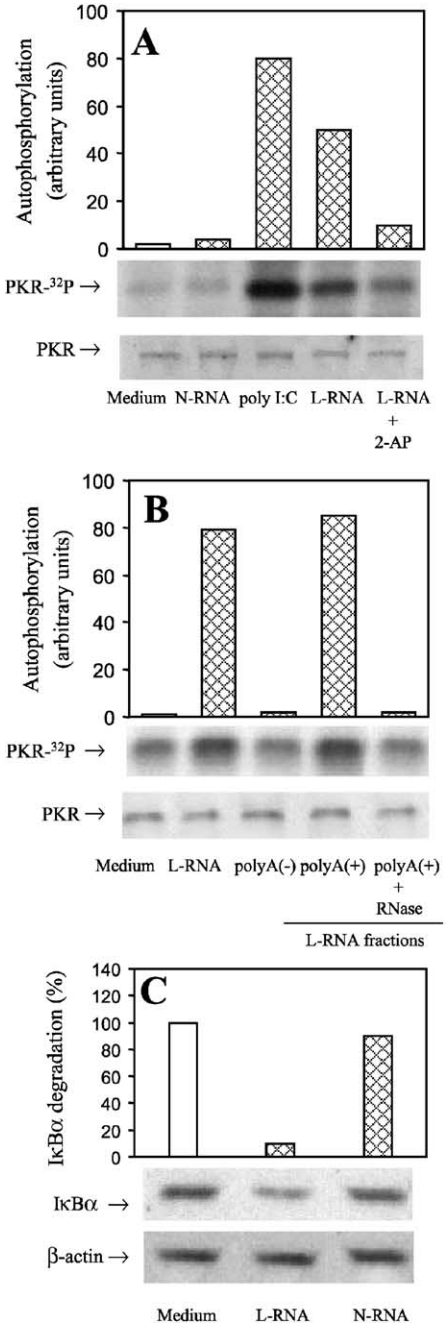

Fig. 1. Effect of L-RNA on PKR autophosphorylation and I-κBα degradation. PKR assays were performed with macrophage extracts (50 μg of protein) and the phosphorylated PKR was purified by affinity chromatography, analyzed by SDS-PAGE and visualized by autoradiography. Quantification of the bands was performed using ImageQuant software, version 3.3, and the results were expressed in arbitrary units. The band corresponding to medium alone was used as control. The same amount of macrophage extract (50 μg of protein) used in PKR assay was also subjected to immunoblotting using anti-PKR as a loading control. (A, upper panel) PKR assay was performed using L-RNA in the presence or absence of the 2-aminopurine (2-AP). Poly (I:C) and N-RNA were used as positive and negative controls, respectively. (A, lower panel) Loading control for PKR assay. (B, upper panel) PKR assay was carried out using poly A(−) and poly A(+) L-RNA fractions. The effect of poly A(+) was evaluated before and after treatment with ribonuclease (RNase). (B, lower panel) Loading control for PKR assay. (C, upper panel) Degradation of I-κBα induced by L-RNA was evaluated by immunoblot analysis using anti-I-κBα and the immunoblot with anti-β-actin was used as loading control (C, lower panel). The percentage of I-κBα degradation was determined using ImageQuant software, version 3.3, and the band corresponding to medium alone was used as control. Results for (A–C) are representative of three independent experiments.

2.6. PKR assay

Macrophages were washed with ice-cold phosphate-buffered saline (PBS) and lysed with three volumes of lysis buffer consisting of 20 mM Tris–HCl (pH 7.6), 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, aprotinin (2 μg/ml), leupeptin (2 μg/ml), 1 mM dithiotreitol, 1% Triton X-100 and 20% glycerol. The lysates were centrifuged at 10 000 × *g* for 20 min and the supernatant (S-10) was stored at −70 °C. The PKR autophosphorylation reactions occurred in a volume of 50 μl containing S-10 (50 μg of protein), 1 mM sodium orthovanadate, 5 mM MgCl, 2 μCi/ml [γ-^{32}P]ATP, 3 mM 2-aminopurine, and polyinosinic:polycytidylic (100 ng/ml), L-RNA (150 μg/ml), poly A(−) L-RNA fraction (150 μg/ml) or poly A(+) fraction L-RNA (2 μg/ml). After incubation at 30 °C for 20 min, the human PKR was purified by using polyinosinic:polycytidylic-agarose and subjected to 8% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. Quantification of PKR phosphorylation was determined by using ImageQuant software, version 3.3 (Molecular Dynamics) and the results expressed in terms of arbitrary units. The same amount of macrophage extract (50 μg of protein) used in PKR assay was also subjected to immunoblotting using anti-PKR (sc #6282, Santa Cruz) as a loading control.

2.7. Western blot analysis

Confluent macrophages were incubated with the N-RNA (75 μg/10^6 cells) or L-RNA (75 μg/10^6 cells) at 37 °C for 1 h in an atmosphere of 5% CO_2. After incubation, the cells were lysed and the proteins were separated by 8% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with anti-I-κBα (sc #1643, Santa Cruz) and anti-β-actin (Boehringer). Protein bands were visualized using the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's instructions. The band corresponding to the β-actin was used as a loading control in all experiments. Quantification of I-κBα degradation was determined using ImageQuant software, version 3.3 (Molecular Dynamics).

3. Results

The first step was to examine the ability of L-RNA to activate PKR from resident macrophages. We found that L-RNA activates PKR in a dose-dependent manner and the peak of activation was achieved at dose of 150 μg of L-RNA/ml (data not shown). Fig. 1A shows results of a typical experiment in which the L-RNA (150 μg/ml) induced PKR autophosphorylation as the polyinosinic/polycytidylic acid which is a classical activator of this protein kinase. We also observed that this effect of L-RNA was abolished by 2-aminopurine, an inhibitor of PKR, and that this protein kinase is not activated by N-RNA.

To determine which L-RNA fraction is responsible for activation of PKR, L-RNA was fractionated on an oligo(dT)-cellulose column. We found that the polyA(+) L-RNA is the active fraction and its activity was abolished by ribonuclease (Fig. 1B).

The next step was to test the effect of L-RNA on I-κBα, an inhibitor of NF-κB. As shown in Fig. 1C L-RNA induce the degradation of I-κBα whereas N-RNA had no effect.

4. Discussion

We have previously demonstrated that L-RNA induces the expression of interleukin-1, interleukin-8 and TNF-α genes in resident macrophages (Ribeiro et al., 1993, 1995). Recently, regulatory RNAs have been shown to regulate gene expression by different mechanisms (Eddy, 2001; Erdmann et al., 2001; Mattick, 2001). Lipopolysaccharide is a potent activator of macrophages and our previous data suggest that lipopolysaccharide induces the synthesis of regulatory RNAs in naive macrophages. The results of the present study support this idea since we found that L-RNA activates PKR which activity was abrogated by its specific inhibitor 2-aminopurine. We also found polyA(+) L-RNA is the fraction responsible for PKR activation and this effect was dependent of the integrity of the polynucleotide chain since its activity was abolished by treatment with ribonuclease.

Several reports have described messenger RNA-like transcripts which are spliced and polyadenylated but have no defined open reading frames (Liu et al., 1997; Erdmann

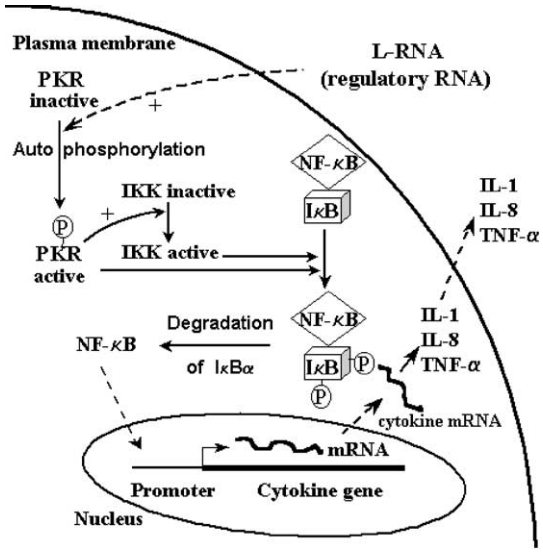

Fig. 2. Model for the mechanism of action of the poly A(+) L-RNA fraction.

et al., 2000). These findings indicate that regulatory RNAs lack protein coding capacity and, therefore, they exert their action at the RNA level (Erdmann et al., 2001; Mattick, 2001). In this context, it is noteworthy that around 98% of all transcriptional output in eukaryotic cells is non-coding RNA (Mattick, 2001).

The most striking observation was the induction of I-κBα degradation by L-RNA which results in NF-κB activation. Heitmeir et al. (1998) also observed I-κBα degradation in macrophages treated with polyinosinic/polycytidylic acid which is a synthetic double-stranded RNA.

On the basis of these data, we suggest a model for the mechanism of action of L-RNA (Fig. 2). According to our model, the incubation of L-RNA with murine macrophages results in its uptake by these cells and thus polyA(+) L-RNA fraction induces PKR activation in the cytoplasm of macrophages. Thererafter, PKR can activate NF-κB either by directly phosphorylating its inhibitor I-κBα (Kumar et al., 1994) or indirectly by activating the I-κB kinase (IKK) complex (Gil et al., 2000). Thus, PKR activation leads to the phosphorylation of I-κBα and its subsequent degradation, thereby freeing the NF-κB to translocate to the nucleus of macrophages. The NF-κB binds to the promoter of interleukin-1, interleukin-8 and TNF-α genes inducing the expression of these genes and the newly synthesized cytokine molecules are secreted into culture medium. Therefore, the polyA(+) L-RNA fraction may act as a regulatory RNA in the phenomenon of induction of the pro-inflammatory cytokines interleukin-1, interleukin-8 and TNF-α.

The results reported here with resting macrophages raises the possibility that polyA(+) L-RNA is also involved in the secretion of pro-inflammatory cytokines by lipopolysaccharide-stimulated macrophages. In this context, our previous showed that macrophages stimulated with lipopolysaccharide release a low molecular RNA (Ribeiro et al., 1993, 1995). These results are consistent with the idea that RNA may be involved as an intercellular modulator of cytokine release from macrophages. Thus, our working hypothesis suggests that resident macrophages, when stimulated by injurious stimuli, in addition to secreting cytokines, are also able to release RNA which may act on the surrounding resting macrophages, stimulating the release of more cytokines. This process may represent amplification of the mechanisms involved in defence responses.

It should be emphasized that most of the focus has been placed on role of the synthetic double-stranded RNA on macrophage activation. Thus, it was reported that polyinosinic/polycytidylic acid activates PKR from macrophages (Gusella et al., 1995) and also induces I-κBα degradation (Heitmeir et al., 1998). However, our work provides evidence for the first time that cellular RNA, obtained from lipopolysaccharide-stimulated macrophages, is also able to activate PKR and NF-κB. Further experimental work is required to isolate and characterize this regulatory L-RNA from the polyA(+) L-RNA fraction.

Acknowledgements

This work was supported by FAPESP (00/09491-7) and CNPq (523412/95-9). We thank Cacilda D.P. Zanon and Zuleica A.S.M. Pereira for the technical assistance. L.R. Souza is the recipient of a fellowship from FAPESP (00/09492-3).

References

Bertolini, M.C., De Lucca, F.L., 1986. Poly(A)-containing RNA from the spleens of mice with Chagas'disease triggers in vitro macrophage resistance to Trypanosoma cruzi. J. Protozool. 33, 81–84.

Clemens, M.J., Elia, A., 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. J. Interferon Cytokine Res. 17, 503–524.

De Lucca, F.L., Sales, V.S.F., Souza, L.R., Watanabe, M.A.E., 2002. Evidence for the involvement of RNA-dependent protein kinase in the induction of cytotoxic T lymphocytes against a synthetic peptide of HIV-1 with regulatory RNA. Mol. Cel. Biochem., in press.

Eddy, S.R., 2001. Non-coding RNA genes and the modern RNA world. Nat. Rev., Genet. 2, 919–929.

Erdmann, V.A., Szymanski, M., Hochberg, A., de Groot, N., Barciszewski, J., 2000. Non-coding, mRNA-like RNAs database Y2K. Nucleic Acids Res. 28, 197–200.

Erdmann, V.A., Barciszewska, M.Z., Szymanski, M., Hochberg, A., de Groot, N., Barciszewski, J., 2001. The non-coding RNAs as riboregulators. Nucleic Acids Res. 29, 189–193.

Gil, J., Alcami, J., Esteban, M., 2000. Activation of NF-kappaB by the dsRNA-dependent protein kinase, PKR involves the I kappa B kinase complex. Oncogene 19, 1369–1378.

Gusella, G.L., Musso, T., Rottschafer, S.E., Pulkki, K., Varesio, L., 1995. Potential requirement of a functional double-stranded RNA-dependent protein kinase for the tumoricidal activation of macrophages by lipopolysaccharide or IFN-α/β, but not IFN-γ. J. Immunol. 154, 345–354.

Heitmeir, M.R., Scarim, A.L., Corbett, J.A., 1998. Double-stranded RNA-inducible nitric oxide synthase expression and interleukin-1 release by murine macrophages requires NF-κB activation. J. Biol. Chem. 273, 15301–15307.

Jagus, R., Joshi, B., Barber, G.N., 1999. PKR, apoptosis and cancer. Int. J. Biochem. Cell. Biol. 3, 123–138.

Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. 18, 621–663.

Kumar, A., Haque, J., Lacoste, J., Hiscott, J., Williams, B.R.G., 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by phosphorylating IκB. Proc. Natl. Acad. Sci. U. S. A. 91, 6288–6292.

Li, J., Petryshyn, R.A., 1991. Activation of the double-stranded RNA-dependent eIF-2 alpha kinase by cellular RNA from 3T3-F442A cells. Eur. J. Biochem. 195, 41–48.

Liu, A.Y., Torchia, B.S., Migeon, B.R., Siliciano, R.F., 1997. The human NTT gene: identification of a novel 17-kb noncoding nuclear RNA expressed in activated CD4+ T cells. Genomics 39, 171–174.

Mattick, J.S., 2001. Non-coding RNAs: the architects of eukaryotic complexity. EMBO Rep. 2, 986–991.

Nussbaum, J.M., Gunnery, S., Mathews, M.B., 2002. The 3′-untranslated regions of cytoskeletal muscle mRNAs inhibit translation by activating the double-stranded RNA-dependent protein kinase PKR. Nucleic Acids Res. 30, 1205–1212.

Passos Jr., G.A.S., De Lucca, F.L., 1991. RNA-mediated transfer of cellular immunity to a synthetic env antigen of the human immunodeficiency virus (HIV-1). Mol. Cell. Biochem. 108, 1–8.

Ribeiro, R.A., De Lucca, F.L., Flores, C.A., Cunha, F.Q., Ferreira, S.H.,

1993. RNA from LPS-stimulated macrophages induces the release of tumor necrosis factor-α and interleukin-1 by resident macrophages. Med. Inflam. 2, 435–442.

Ribeiro, R.A., Flores, C.A., Cunha, F.Q., Ferreira, S.H., De Lucca, F.L., 1995. Partial characterization of the RNA from LPS-stimulated macrophages that induces the release of chemotactic cytokines by resident macrophages. Mol. Cell. Biochem. 148, 105–113.

Tak, P.P., Firestein, G.S., 2001. NF-κB: a key role in imflammatory diseases. J. Clin. Invest. 107, 7–11.