Cytotechnology (2016) 68:2659–2666
DOI 10.1007/s10616-016-9990-1

ORIGINAL ARTICLE

Interleukin-1β effect on the endogenous ADP-ribosylation and phosphorylation of eukaryotic elongation factor 2

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Received: 17 August 2015 / Accepted: 26 May 2016 / Published online: 10 August 2016

Abstract Eukaryotic elongation factor 2 (eEF2) plays an important role in eukaryotic polypeptide chain elongation. Adenosine diphosphate (ADP)-ribosylation is a post-translational modification reaction that catalyzes the transfer of ADP-ribose group to eEF2 and this causes the inhibition of protein synthesis. Indeed, in the absence of diptheria toxin, endogenous ADP-ribosylation can occur. eEF2 is phosphorylated by eEF2 kinase which prevents binding to ribosomes thus inhibiting its activity. Increase in endogenous ADP-ribosylation level approximately 70–75 % was observed in IL-1β treated HUVECs. Moreover, a 70 % rise of phosphorylation of eEF2 was measured. Alteration of endogenous ADP-ribosylation of eEF2 activity was related with cellular mono-ADP-ribosyltransferases (ADPrT). Increment of endogenous ADP-ribosylation on eEF2 did not seem to occur as a direct effect of IL-1β; it arises from the activation of ADPrT. This 2.5 fold increase was abolished by ADPrT inhibitors. Due to these post-translational modifications, global protein synthesis is inhibited. After dephosphorylation of phospho-eEF2, around 20 % increase in protein synthesis was observed. In conclusion, systemic IL-1β has an important role in the regulation of global protein synthesis.

Keywords Diphtheria toxin · Endogenous ADP-ribosylation · Eukaryotic elongation factor 2 · Interleukin-1β · Phosphorylation · Protein synthesis

Abbreviations

ADPR	ADP-ribose
DTx	Diphtheria toxin
eEF2	Eukaryotic elongation factor 2
HUVEC	Human umbilical vein endothelial cells
IL-1β	Interleukin-1β
NAD^+	Nicotinamide adenine dinucleotide

Introduction

Eukaryotic elongation factor 2 (eEF2) is responsible from eukaryotic polypeptide chain elongation, which promotes translocation in this process (Bermek 1978). eEF2 is a 97 kDa protein of GTP binding protein family, which catalyses the translocation of peptidyl-tRNA on the ribosome (Merrick and Nyborg 2000). eEF2 is an important site for cellular control mechanisms through post-translational modification (ADP-ribosylation, phosphorylation). It provides the

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eukaryotic cell with a unique site of attack for *Pseudomonas aeroginosa* exotoxin A and diphtheria toxin (DTx) (Collier 1967; Iglewski and Kabat 1975; Varol et al. 2012). ADP-ribosylation of the diphthamide residue (H^{715}) of eEF2 causes inactivation of eEF2. It has previously been argued that the toxin effect may simulate a regular cellular control mechanism (Collier 1975). Indeed, an endogenous ADP-ribosyltransferase-specific activity for eEF2 has been shown to exist in mammalian systems, (Lee and Iglewski 1984; Sitikov et al. 1984), which depicts inherent property of eEF2 (Sayhan et al. 1986). It has been suggested that the binding of free ADP-ribose, DTx and endogenous transferase catalysed ADP-ribosylations in the presence of NAD^+ are notable reactions (Bektaş et al. 2005, 2006). Endogenous ADP-ribosylation is reported to be increased in aging and oxidative stress (Bektaş et al. 2005; Ayala et al. 1996; Parrado et al. 1999; Argüelles et al. 2013). Auto-ADP-ribosylation reaction of cholix toxin involves the intramolecular transfer of ADP-ribosyl moiety to multiple arginine residues located around the NAD^+ binding pocket as well as distal sites on the opposite face of the enzyme. It was also reported that cholix toxin auto ADP-ribosylates eEF2 (H^{715R}) mutant in which the post-translationally modified diphthamide at His^{715} was replaced by arginine (Sung and Tsai 2014).

Another line of research reveals that eEF2 can also be specifically phosphorylated by a Ca^{2+}/calmodulin dependent kinase (protein kinase III or eEF2 kinase) (Nairn et al. 1985), and dephosphorylation consists in the activity of protein phosphatase 2A (Everett et al. 2001). Phosphorylation of eEF2 (T^{56}) inhibits its activity by reducing its affinity to ribosomes, resulting in inhibition of eukaryotic protein synthesis (Ryazanov and Spirin 1990). Nutrients and growth factors are known as the main regulators of the phosphorylation of eEF2. They activate mTOR (mammalian target of rapamycin) that causes the S6 kinase mediated inhibition of eEF2 kinase and results in the reduction in the phosphorylation of eEF2 and increase in protein synthesis. eEF2 is regulated by several post-translational mechanisms such as ADP-ribosylation, phosphorylation, and protein–protein interactions. (Argüelles et al. 2013). The presence of both modified variants of eEF2 in cell lysates, (Celis et al. 1990; Riis et al. 1990) increase in relative proportions of the phosphorylated variant of eEF2 during mitosis (Celis et al. 1990) and decrease in levels of ADP-ribosylatable eEF2 in G_0/G_1 arrested cells have been reported (Riis et al. 1990).

Interleukin-1β (IL-1β) is a proinflammatory cytokine produced by macrophages and monocytes. It is a potent mediator of fever, pain, and inflammation (Contassot et al. 2012). IL-1β affects almost all cell types and its secretion is increased significantly in the pathogenesis of several diseases (Church et al. 2008). It also stimulates endogenous (mono) ADP-ribosylation in cardiomyocytes (Jäger et al. 2011).

In this study, increase in endogenous ADP-ribosylation and phosphorylation activity of eEF2 was observed in IL-1β treated HUVECs with autoradiogram and western blot (WB) techniques. Consequently, increase of endogenous ADP-ribosylation of eEF2 can be thought with effect of IL-1β on cellular ADPrT activity instead of direct effect of eEF2.

Materials and methods

Materials

All reagents were purchased from the Sigma (St. Louis, MO, USA). [^{14}C]Phe, and [adenosine-^{14}C]NAD^+ was purchased from Perkin Elmer Life Sciences (Waltham, MA, USA); anti IL-1β monoclonal antibody (mouse) from Abnova (Walnut, CA, USA) anti-eEF2 polyclonal antibody (rabbit) from Pierce (Waltham, MA, USA); anti-eEF2 (phospho T^{56}) polyclonal antibody from Abcam (Cambridge, UK); and the DTx from the Calbiochem (San Diego, CA, USA). Rat liver ribosomes, eEF2 and eEF1 were prepared as previously described (Bektaş et al. 1994).

Methods

Cell culture and treatment of cells with interleukin-1β

HUVECs (ATCC-CRL-1730) were distributed in 35 mm diameter culture wells (10^6 cells/well) and were propagated in DMEM-F12 medium (HyClone, Waltham, MA, USA) that contained 15 % heat-inactivated fetal bovine serum (FBS; Gibco; Invitrogen Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin sulfate with IL-1β (Millipore, Billerica, MA, USA) in the medium at 37 °C with 5 % CO_2. After the indicated periods of time,

cells were collected using a cell scraper (Greiner Bio-One, Kremsmünster, Austria) and washed three times in phosphate-buffered saline (PBS). The cells were homogenized in 1 % (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, in the presence of protease inhibitors (Complete Tablets, Roche Applied Science, Mannheim, Germany). The protein concentrations of the homogenates were determined (DC Protein Assay, Biorad, Hercules, CA, USA). Cell extracts were first clarified by ultracentrifugation for 2 h at 100,000×*g*. The cytosolic fractions (S_{100}) were then assayed in endogenous ADP-ribosylation, phosphorylation and polyPhe synthesis. A pull-down assay kit (Pierce, Waltham, MA, USA) was used to remove IL-1β and eEF2 from cell lysates. The anti-IL-1β and anti-eEF2 were labeled with sulfo-NHS-LC biotin from Pierce at a molar ratio of 1/50 by incubating at room temperature for 1 h (Bektaş et al. 2009). The labeled antibodies were dialyzed overnight against PBS. Antibody bound proteins (IL-1β or eEF2) were treated with streptavidin-coated beads and collected by centrifugation. IL-1β and eEF2 quantities of cell lysates were determined using ELISA or ADP-ribosylation.

Preparation of eEF2

eEF2 was purified from the rat livers as previously described (Bektaş et al. 2006). Briefly, rat liver was homogenized in medium A (50 mM Tris–HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 7 mM 2-mercaptoethanol and 250 mM sucrose) with homogenizer. The homogenate was performed to differential centrifugation: following the initial centrifugation for 20 min at 30,000×*g*, the postmitochondrial supernatant was centrifuged for 2 h at 130,000×*g*. eEF2 was isolated from the post-ribosomal supernatant by adsorption to hydroxyapatite and subsequent elution. eEF2 was eluted with between 50 and 150 mM potassium phosphate (pH 7.4). eEF2 was purified using chromatography on DEAE-cellulose (DE52, Whatman, Maidstone, UK) and eluted with between 15 and 65 mM KCl in 50 mM Tris HCl, pH 7.4, containing 7 mM 2-mercaptoethanol and 0.1 mM EDTA. For final purification, eEF2 was subjected on phosphocellulose (P11, Whatman), and eEF2 was eluted with between 50 and 250 mM KCl, potassium phosphate (pH 6.8), 7 mM 2-mercaptoethanol. eEF2 amount was determined by diphtheria toxin-dependent ADP-ribosylation and electrophoretic (Western blot and autoradiograph) analysis.

ELISA

ELISA was performed as described by Engvall and Perlmann (1971); 100 µl samples were added to the micro-well plates. The wells were then incubated; antibody buffer (PBS containing 1 % bovine serum albumin, and 0.1 % Tween-20) was added at 37 °C for 30 min to block nonspecific binding sites. The wells were then incubated in parallel with anti-IL-1β and anti-eEF2 in 1/1000 dilution in antibody buffer for 2 h at 37 °C. After washing with PBS, bound antibody was detected using a secondary horseradish peroxidase-conjugated anti-mouse IgG antibody (1/1000 in antibody buffer) for 1 h at 37 °C. After a final wash in PBS, the peroxidase activity was detected in line with the instructions specified in OPD reagent kit (Bio-Rad, Hercules, CA, USA). Amounts of IL-1β and eEF2 were determined by reading absorbance at 490 nm.

ADP-ribosylation

ADP-ribosylation was carried out for 10 min at room temperature in 20 µl reaction mixtures that contained 50 mM Tris–HCl, pH 7.4, 7 mM 2-Mercaptoethanol, 2 µM [^{14}C]NAD, and 100 µg/ml of DTx (Bektaş et al. 2006). Endogenous transferase-dependent ADP-ribosylation was assayed under the same conditions in the absence of diphtheria toxin and the incubation time was extended to 5–120 min at 20 °C. Following incubation, 10 µl aliquots were applied to GF/A (Whatman) filters that were washed successively in cold trichloroaceticacid (TCA), ether-ethanol (v/v: 1/1) and ether. After drying, the filters were transferred to vials containing 2.5 ml of 0.4 % 2,5 diphenyloxazole in toluene and TCA-precipitated radioactivity was determined in a liquid scintillation counter (Packard TriCarb 2100 TR, Perkin Elmer Life Sciences, Inc., Zaventem, Belgium). Alternatively, cells were lysed in lysis buffer (0.5 % Triton X-100, 1 mM PMSF, 1 mM EDTA, 10 mM Tris–HCl; pH: 7.4 and 1:100 protease inhibitor cocktail), and cell lysates (10 µl containing 50 µg of protein) were incubated with 10 µM 6-biotin-17-NAD (Trevigen, Gaithersburg, MD, USA) and 1 µg diphtheria toxin for 30 min at 20 °C. Samples were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and ADP-ribosylated eEF2 was detected with anti-biotin (SIGMA) and Streptavidin-AP conjugated antibody (Biorad) (Argüelles et al. 2013).

Western blot and autoradiographic analysis

SDS–PAGE was performed as described by Laemmli (1970). The proteins were stained with Coomassie blue and destained in 10 % acetic acid and 50 % methanol, and the dried gel slabs were exposed to Kodak X-Omat K films at −70 °C. For visualization of the protein bands corresponding to phosphorylated eEF2 or dephosphorylated eEF2 proteins that had been separated using electrophoresis were transferred to nitrocellulose membranes and then were treated with TBST (Tris-buffered saline-Tween) and 0.5 % BSA for 1 h. The membranes were incubated with eEF2 or phosphorylated eEF2-specific (phospho T^{56}) antibody and then with alkalin phosphatase conjugates of anti-rabbit IgG antibody. After three washes with TBST, NBT-BCIP was used for detection of the protein bands. eEF2 was dephosphorylated with protein phosphatase (PP2A) (Sigma), 5 μg/ml in 30 μl of reaction buffer for 60 min at 30 °C.

Protein synthesis measurement

In vitro protein synthesis (polyPhe synthesis) was performed as described by Bektaş et al. (2011). Reaction mixtures (50 μl) containing about 20 pmol ribosomes, 40 μg/ml eukaryotic elongation factors (eEF1 and eEF2), 100 μg/ml of polyU, 300 μg/ml tRNA (*E. Coli*), charged during the reaction by use of DE52 chromatography (Whatman) fractionated S_{100} (*E. coli*) as a source of AA-tRNA synthetases, 2 μM of [^{14}C]Phe, 6 mg/ml of creatine kinase/creatine phosphate, 1.5 mM ATP and GTP. Cytosolic fractions (S_{100}) were used as a source of eEF2. The incubations were performed for 3 min at 37 °C, and TCA-precipitated radioactivity was determined as above with the inclusion of a prior 5 % TCA treatment for 5 min at 90 °C.

Data analysis

Data were expressed as mean ± standard deviation of three independent experiments and statistically analyzed using SPSS version 17.0. Statistical analysis was performed using the unpaired Student's *t* test and ANOVA followed by Bonferroni test. Statistical significance was accepted at p values <0.05. Western blots and autoradiographs were quantified by using ImageJ 1.32 software.

Results

Endogenous ADP-ribosylation of eEF2 with IL-1β

ADP-ribosylation of eEF2 was analyzed with [^{14}C]NAD^+ and DTx in the absence and presence of increasing amounts IL-1β for 12 hours. 75 % decrease in ADP-ribosylation was observed with high amount of IL-1β treatment (Fig 1a). Reduction in the ADP-ribosylation of eEF2 with DTx shows that endogenous ADP-ribosylation occurred with ADPrT in presence of IL-1β (Bektaş et al. 2005; Jäger et al. 2011). Endogenous ADP-ribosylation was increased to 45 % in 30 min and 52 % in 60 min in cell lysates treated with 100 U/ml IL-1β for 12 h (Fig. 1b). The total amount of eEF2 remained constant both in the presence and absence of IL-1β (Fig. 1c). In addition to scintillation analysis in Fig. 1a, radioactive eEF2 was shown via autoradiography after SDS–PAGE electrophoresis (Fig. 1d). These results support the data that are shown in the presence of DTx in Fig. 1a.

Direct effect of IL-1β on ADP-ribosylation of eEF2

Endogenous ADP-ribosylation was performed with eEF2 purified from rat liver. There was no direct effect of IL-1β on ADP-ribosylation (Fig. 2). Effect of IL-1β on endogenous ADP-ribosylation in purified eEF2 and HUVEC lysates (S_{100}) was examined and results are presented in Table 1. ADP-ribosylation was measured 4.5 pmol with eEF2 alone, however, it increased to 11.2 pmol when IL-1β treated S_{100} lysates were added. Cibacron Blue, and isonicotinic acid hydrazide (INH) are the inhibitors of the endogenous ADP-ribosylation. These reagents considerably reduced the endogenous transferase activity on endogenous transferase dependent ADP-ribosylation of eEF2, to an extent of 65 % (Cibacron Blue, INH combination) and 70 % (L-Arginine).

Phosphorylation of eEF2 with IL-1β

In addition to ADP-ribosylation assay, phosphorylation of eEF2 was visualized with SDS–PAGE, WB and densitometric analysis. It was induced by about 4–5 fold with increasing concentrations of IL-1β in a 12 hours incubation (Fig. 3a). Following phosphorylation of eEF2, the reaction was widely inhibited by PP2A (Fig. 3b). The eEF2 amount was determined both in ELISA and ADP-ribosylation.

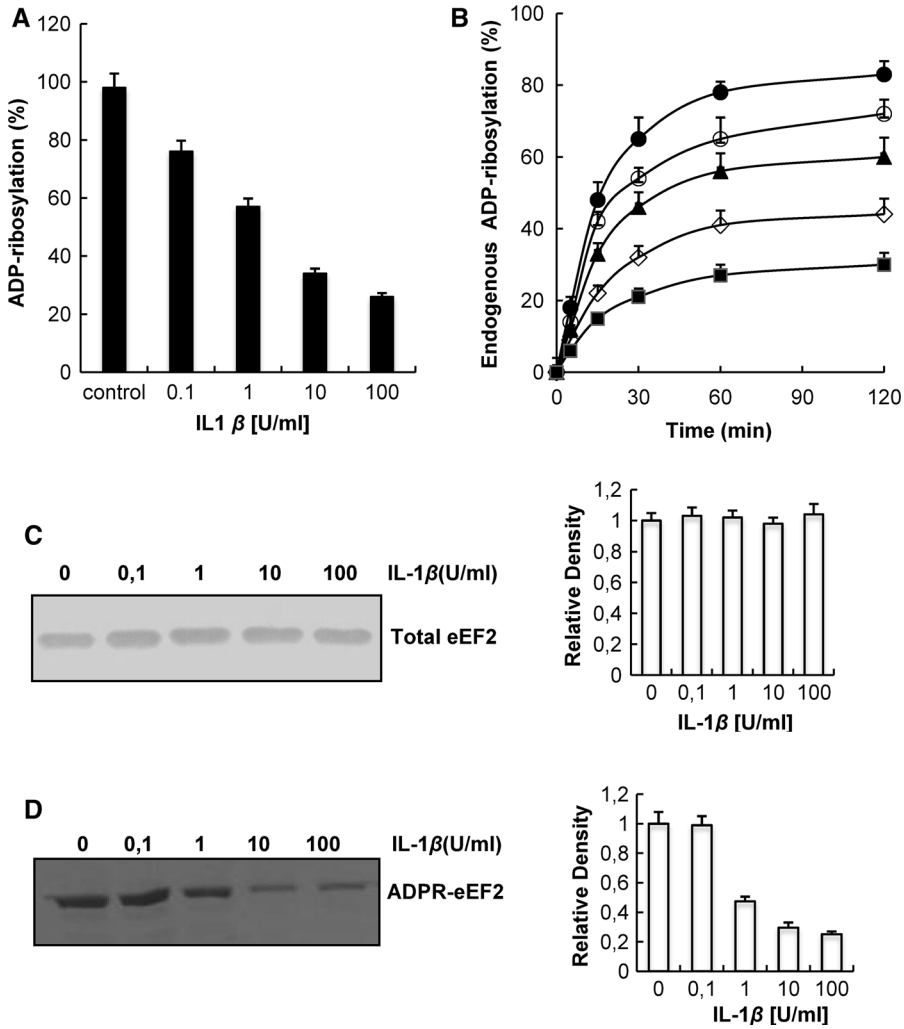

Fig. 1 Effect of IL-1β on ADP-ribosylation of eEF2. Cell lysates (S_{100}) were prepared with IL-1β treatment (12 h incubation). 1 mg S_{100} protein was ADP-ribosylated in the presence of 5 µM [^{14}C]NAD and 100 µg/ml DTx. **a** ADP-ribosylation in the presence of IL-1β after scintillation. Control is IL-1β free. **b** Endogenous ADP-ribosylation of eEF2 in cell lysate (1 mg S_{100} proteins). (*Square*) control (IL-1β free), (*diamond*) 0.1 U/ml, (triangle) 1 U/ml, (*open circle*) 10 U/ml, (*filled circle*) 100 U/ml IL-1β. Percentages are compared with ADP-ribosylation of eEF2 with DTx in 1 mg S_{100} proteins. **c** Western blot image of total eEF2 (2 mg S_{100} proteins). **d** Autoradiogram of ADP-ribosylation in the presence of IL-1β (2 mg ADP-ribosylated S_{100} protein). Graphs on the *right side* show the analysis of western blot and of the autoradiogram, respectively, using ImageJ software. The resulting values are a measure of the relative density of each sample, compared to the IL-1β free (0 U/ml)

IL-1β effect on polyPhe synthesis

In vitro protein synthesis assay was performed as described in Materials and methods. The supplementation with 0.1, 1, 10, 100 (U/ml) IL-1β inhibited protein synthesis by 25 %, 38 %, 57 % and 60 %, respectively. In the presence of dephosphorylated eEF2, inhibition of polyPhe synthesis was reduced by 10–15 % (Fig. 4) in comparison to the eEF2 conditions. This recovery is significant according to polyPhe synthesis of IL-1β treated samples.

Discussion

An impairment of eEF2 by IL-1β activity could be pathogenically relevant because IL-1β plays an important role in numerous systemic inflammatory diseases. In the present study, we report that IL-1β

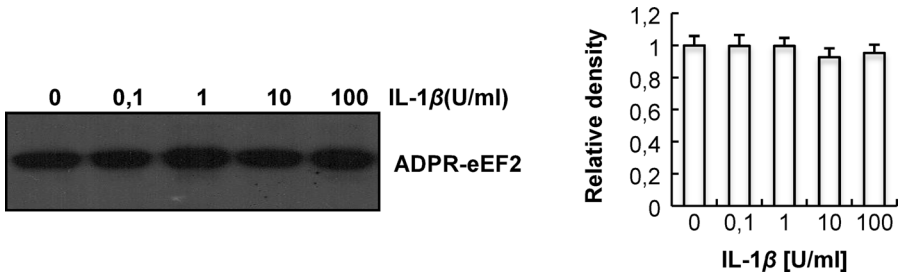

Fig. 2 Direct effect of IL-1β on ADP-ribosylation of eEF2. Autoradiograph of ADP-ribosylation in the presence of 5 μM [^{14}C]NAD, 100 μg/ml DTx and 20 pmol (2 μg) eEF2 isolated from rat liver incubated with IL-1β (12 h). The graph on the *right side* shows the analysis of western blot using ImageJ software. The resulting values are a measure of the relative density of each sample, compared to the IL-1β free (0 U/ml)

Table 1 IL-1β effect on transferase activity

Treatment	Endogenous ADP-ribosylation (pmol)/30 min
S_{100} (0.5 mg proteins)	1.4 ± 0.07
eEF2 (20 pmol)	4.5 ± 0.3
eEF2 + IL-1β (10 U/ml)	6.5 ± 0.34
eEF2 + S_{100}	6.3 ± 0.32
eEF2 + S_{100}, IL-1β	11.2 ± 0.8
eEF2 + S_{100}, IL-1β + Cibacron (1 mM), INH (10 mM)	5.8 ± 0.26
eEF2 + S_{100}, IL-1β + L-Arginine (10 mM)	3.8 ± 0.4

Effect of different compounds on endogenous ADP-ribosylation was performed with 10 μM [^{14}C]NAD for 30 min in the presence of IL-1β (12 h incubation)

INH isonicotinic acid hydrazide

induces the endogenous ADP-ribosylation and phosphorylation of eEF2 in HUVECs. Activity of eEF2 was reduced in the presence of IL-1β similar to the DTx effect on protein synthesis.

Elevation in eEF2 phosphorylation was considerable and this increase of endogenous ADP-ribosylation of eEF2 can be considered as an inherent result of ADPrT, and not as a direct effect of IL-1β. Activation of p90RSK phosphorylates and inactivates the elongation factor 2 kinase (eEF2K) in muscle cells. This inhibition causes a reduction in the inhibitory phosphorylation of eEF2. The dephosphorylated form of eEF2 can catalyse translocation and promotes protein synthesis (Wang et al. 2001; Pelosi et al. 2014). Moreover, there was an inhibition in protein synthesis after the effect of phosphorylation was extinguished, which correlates with previous in vivo research (Pönicke et al. 2001). Studies indicated that intracellular eEF2 was ADP-ribosylated endogenously at around 70–80 % during IL-1β treatment. Moreover remaining unmodified (native) eEF2 (20–30 %) can carry on the protein synthesis process at a steady state, however, it takes a much longer time. Protein synthesis rate can work as a limiting factor and therefore it can prioritize induction or inhibition of various proteins.

Cibacron Blue and INH was used because of their relatively strong inhibitory effect on endogenous ADP-ribosylation. The combination of Cibacron Blue and INH decreased endogenous transferase activity by blocking ADP-ribose binding. L-Arginine also proved to be a strong inhibitor of endogenous transferase dependent ADP-ribosylation. It has been reported that arginine residues inhibit ADP ribosylation of eEF2 in the presence of cholix toxin (Sung and Tsai 2014). This was also shown for DTx (Lory et al. 1980) but using different experimental conditions. Endogenous ADP-ribosylation in presence of arginine and other residues is a rather slow reaction (30 min–1 hour). Moreover it requires high concentrations of NAD (50–100 μM). On the other hand, in presence of DTx and 2 μM NAD ADP-ribosylation of eEF2 happens within 10 minutes. In this context, 5–10 minutes is an adequate time to ADP-ribosylate dipthamide residue of eEF2.

In this context, it is clear that in contrast to reactions catalyzed by endogenous ADPrTs, the cytotoxic efficiency of ADP-ribosylating toxins appears to be extremely high. It has been shown that DTx is an extremely lethal agent with one single molecule being sufficient for induction of eukaryotic cell death (Collier 1975). Cellular ADP-ribosylation by ADPrTs may be a

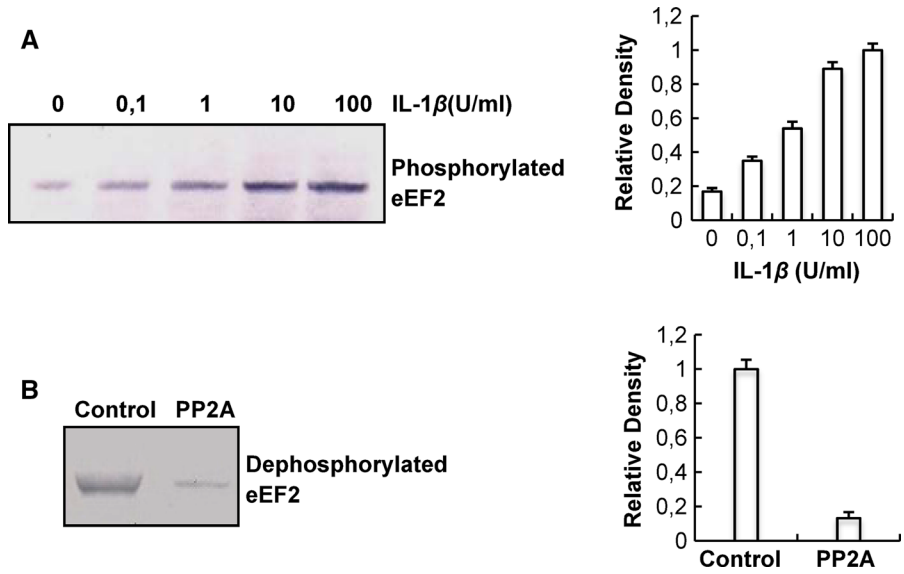

Fig. 3 Effect of IL-1β on phosphorylation of eEF2. **a** Western blot analysis of cell lysates (10 mg S_{100} proteins) in the presence of increasing concentrations of IL-1β (12 h incubation) using anti-phospho T^{56} eEF2 antibody. The graph on the *right side* shows the analysis of western blot using ImageJ software. The resulting values are a measure of the relative density of each sample, compared to the 100 U/ml IL-1β. **b** Phosphorylated-eEF2 (10 mg S_{100} proteins) in the presence of 100 (U/ml) IL-1β with using PP2A (5 mg/ml) for dephosphorylation. The graph on the *right side* shows the analysis of western blot using ImageJ software. The resulting values are a measure of the relative density of each sample, compared to the 100 U/ml IL-1β (Control)

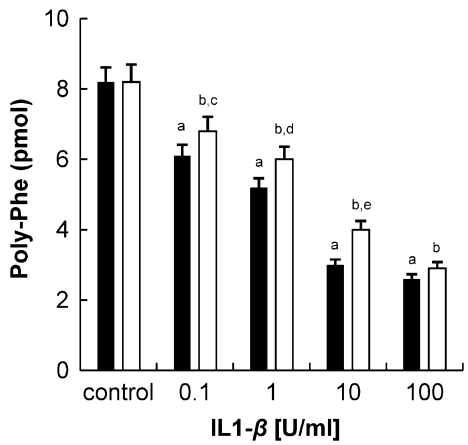

Fig. 4 Effect of IL-1β on in vitro protein synthesis. PolyPhe synthesis was performed in the presence of IL-1β (0.1–100 U/ml) for 12 hours and S_{100} protein (2 mg) as source for eEF2 (*black bars*). Control is IL-1β free. *Light bars* show polyPhe synthesis in presence of dephosphorylated eEF2. All data are representative of three independent experiments and statistical significance was measured using Student *t* test. [a]Statistically different from 0 U/ml IL-1β treatment group ($p < 0.05$). [b]Statistically different from 0 U/ml IL-1β and 5 µg/ml PP2A treatment group ($p < 0.05$). [c]Statistically different from 0.1 U/ml IL-1β treatment group ($p < 0.05$). [d]Statistically different from 1 U/ml IL-1β treatment group ($p < 0.05$). [e]Statistically different from 10 U/ml IL-1β treatment group ($p < 0.05$)

dynamic process that affects eEF2 function under certain conditions. This is supported by recent studies that ADPR-eEF2 becomes up-regulated in cardiomyocytes when treated with IL-1β (Jäger et al. 2011) and in neurons under oxidative stress (Argüelles et al. 2013). In line with this result, a link may exist between oxidative stress protection, diphthamide, and eEF2 function. Post-translational modifications of eEF2 under oxidative stress render complex ribosome, mRNA, and eEF2 interactions which are required for global protein synthesis reorganization (Argüelles et al. 2014). Inflammation also reveals similar changes. Several studies showed that, eEF2/p53 interaction was modified with oxidative stress conditions in primary hippocampal neurons (Argüelles et al. 2013). eEF2 can be regulated by endogenous ADP-ribosylation and protein–protein interactions and these regulations can be important in inflammation increase warranting further investigation.

In conclusion, the IL-1β affect is a systemic and important issue with respect to regulation of global protein synthesis.

Acknowledgments This work was supported by the Research Fund of the Istanbul University (Grant 31384).

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