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An endogenous redox molecule, thioredoxin, regulates transactivation of epidermal growth factor receptor and activation of NF-κB by lysophosphatidic acid

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Abstract Lysophosphatidic acid (LPA) is the smallest and simplest of all the glycerophospholipids that activates a specific GTP-binding protein coupled receptor to evoke multiple cellular responses. In this paper, we have demonstrated that LPA stimulates nuclear factor (NF)-κB-dependent gene induction in a neuronal cell line, NG108-15 and that this is under redox regulation by an endogenous molecule, thioredoxin. We also have shown that redox-sensitive transactivation of epidermal growth factor receptor by LPA confers NF-κB activation and small GTPase proteins are involved in this pathway.

Key words: Lysophosphatidic acid; Nuclear factor κB; Thioredoxin; Redox

1. Introduction

Lysophosphatidic acid (LPA; 1-acyl-2-lyso-*sn*-glycero-3-phosphate) is a naturally occurring, water-soluble glycerophospholipid that exhibits striking hormone- and growth factor-like activities [1]. LPA is normally in serum and acts on fibroblast, endothelial cells, and smooth muscle cells [2]. Cellular responses to LPA include changes in cell shape, chemotaxis, proliferation, and differentiation [1]. In neuronal cells, it influences neurite extension and neurotransmitter release. It is now well established that LPA induces cellular responses by binding to a seven-transmembrane domain motif characteristic of GTP-binding regulatory protein (G protein)-coupled receptors (GPCRs) that is coupled to Gi, Gq, G12, and G13 subfamilies of heterotrimeric G protein [1,3]. It has been observed that LPA, as well as other agonists of GPCRs, induces tyrosine phosphorylation of several signalling proteins in diverse cell types. It was reported that LPA and some other agonist of GPCRs, such as thrombin and bradykinin, transactivate the epidermal growth factor receptor (EGFR) [4]. Although a line of evidence clearly demonstrates the significance of this transactivation pathway for LPA-induced mitogen-activated protein kinase (MAPK) activation, there are only a few reports on the other signalling pathways such as nuclear factor (NF)-κB activation, which has been shown to play a pivotal role in inflammation and life and death of cells.

The binding of ligands to certain GPCRs leads to activation of NF-κB. Ligands include serotonin, platelet-derived growth factor, thrombin, and bradykinin [1]. In addition, LPA also promotes activation of NF-κB in a fibroblast cell line, Swiss 3T3 cells [5] and in primary endothelial cells [6]. Details of mechanism from GPCR to NF-κB are largely unknown, although the signalling pathway to the activation of NF-κB from inflammatory cytokines is one of the most extensively studied [7]. As well as tumor necrosis factor, several extracellular stimuli, including oxidative stress, activate NF-κB [8,9]. It has been reported that antioxidants, such as *N*-acetyl-L-cysteine (NAC) and pyrolidine dithiocarbamate (PDTC), suppress the NF-κB activation in certain cells, indicating that the activation of NF-κB is subject to redox regulation [7,9]. It has been demonstrated that LPA stimulates reactive oxygen intermediates (ROIs) production in HeLa cells and antioxidants such as NAC or PDTC inhibit LPA-induced NF-κB activation [5]. However, NAC and PDTC are all synthesized compounds and the details of mechanisms or target molecules are not well described.

Thioredoxin (TRX) is a 12 kDa endogenous protein [10,11]. The TRX system including TRX reductase and NADPH, is involved in the thiol-regulated intracellular redox system as well as the glutathione system [11]. TRX has been shown to regulate various cellular functions by modulating the redox state of proteins and control the activities of various types of transcription factors including NF-κB, AP-1 and CREB [12–15]. TRX participates in redox reactions by reversible oxidation of its active center dithiol to a disulfide and has peroxidase activity such as catalase [11,16]. Thus TRX is involved in many thiol-dependent cellular processes including proliferation and apoptosis, and gene expression and signal transduction [13].

In this study, using a neuronal cell line, NG108-15, we demonstrate that LPA transactivates EGFR, that this transactivation confers NF-κB activation by LPA and that an endogenous thiol regulating molecule, TRX suppresses LPA-elicited transactivation of EGFR and activation of NF-κB. In addition, we show that certain alpha subunits of heterotri-

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Abbreviations: LPA, 1-acyl-2-lyso-*sn*-glycero-3-phosphate (lysophosphatidic acid); G protein, GTP-binding protein; GPCR, G protein-coupled receptor; ROI, reactive oxygen intermediate; TRX, thioredoxin

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meric G protein and Rho family small G proteins are playing significant roles in this redox-sensitive pathway.

2. Materials and methods

2.1. Cell lines and reagents

Mouse neuroblastoma x rat glioma hybrid NG108-15 cells [17] are from Dr. Haruhiro Higashida, Kanazawa University. NG108-15 cells were maintained in DMEM supplemented with 5% FBS, 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine at 37°C under a humidified atmosphere of 5% CO_2. LPA and NAC were purchased from Sigma. Recombinant human EGF was from Boehringer Mannheim GmbH. Monoclonal antibodies raised against EGF receptor (E12020) and activated EGF receptor (E12120) were from BD Transduction Laboratory. Tyrphostin AG1478, tyrphostin A25, and pertussis toxin (PTX) were from Calbiochem.

2.2. Expression plasmids

Expression plasmids of wild-type human TRX and its redox-inactive mutant, pcDNA3-TRX-wt and pcDNA3-TRX-dm, were described elsewhere [14]. An expression plasmid of human IκBα32A/36A mutant, pRcIκBα32A/36A [18], was from Dr. Nancy R. Rice, NCI, Frederic. Constitutively activated G proteins, pcDNA3-Gq Q209L, pcDNA3-G12 Q229L, and pcDNA3-G13 Q226L [19] were kindly provided by Dr. Manabu Negishi, Kyoto University. Expression plasmids of small G proteins, Rac1 [20] and RhoA were gifts from Dr. Kozo Kaibuchi, NAIST and Dr. Shu Narumiya, Kyoto University respectively. pNF-κB-Luc and pSV-β-galactosidase were from Stratagene and Promega, respectively. An expression vector of GFP-fused EGF receptor, in which the GFP moiety is fused to the carboxyl-terminus of EGFR, pEGFR-EGFP [21] was kindly provided by Dr. Alexander Sorkins, University of Colorado, CO, USA. pEGFP-N3 was obtained from Clontech.

2.3. Reporter gene assay

NG108-15 cells were plated in 24-well plates at a density of 5×10^4 cells per well. The Fugene 6 reagent (Roche) was used for the transfection procedure. Total amount of DNA was adjusted equally with pcDNA3. After treatment, the cells were harvested and the luciferase activity was determined using an assay system (Promega) with a luminometer, Lumat LB9507 (Berthold). The relative fold induction of luciferase activity was calculated after normalization dividing the luciferase activity by β-galactosidase activity [14,15]. Each experiment was done at least two times in triplicate and data shown are representatives of them. The results are the means ± S.D. and presented as relative luciferase activity over the baseline seen with the mock transfectant.

2.4. Western blotting

After treatments, cells were lysed with a buffer (5 mM HEPES; 250 mM NaCl, 10% (v/v) glycerol, 0.5% Nonidet P-40, 100 μM Va_3VO_4 supplemented by Complete protease inhibitor cocktail, Roche). 50 μg of lysates were separated on 7.5% SDS–PAGE gels. Western blotting analysis was performed following a protocol described previously [14].

2.5. Statistical analysis

Statistical significance between two groups was tested using the unpaired Student's t test. Differences were considered statistically significant at a value of $P<0.05$.

3. Results

3.1. Induction of NF-κB-dependent luciferase activity by LPA is redox-sensitive

To examine effect of LPA on NF-κB-dependent transcriptional activity, we treated NG108-15 cells with LPA. As shown in Fig. 1A, 20 μM of LPA significantly induced activation of NF-κB. Overexpression of mutant form of IκBα, IκBα32A/36A inhibited the LPA-induced activation. Overexpression of wild-type TRX in a plasmid-dose responsive manner or treatment with NAC inhibited the LPA-induced activation (Fig. 1B). A mutant-type TRX, which has no reducing activity, did not suppress the activation. Interestingly, TRX expression did not inhibit IKKα-induced NF-κB activation (data not shown) in NG108-15 cell as well as in HEK293 cells [22].

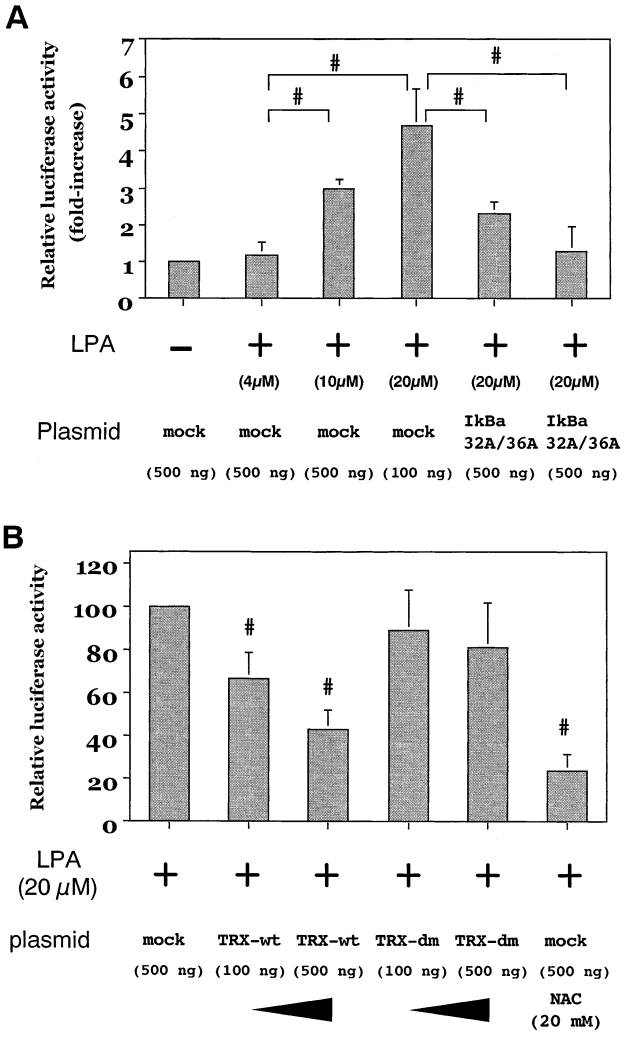

Fig. 1. Induction of NF-κB-dependent luciferase activity by LPA is TRX-sensitive. NG108-15 cells were transfected with 250 ng of pNF-κB-Luc, 100 ng of pSV-β-galactosidase, and an indicated amount of pRc-IκBα32A/36A, pcDNA3-TRX-wt or pcDNA3-TRX-dm. At 12 h after transfection, cells were treated by LPA with or without NAC pretreatment. After 6 h incubation, cells were harvested and underwent the luciferase assay following the protocol described in Section 2. The results are the means ± S.D. and presented as fold increase in luciferase activity over the baseline seen with the mock transfectant without treatment (A) or as percent induction over the mock transfectant with LPA treatment (B). This is a representative of two independent experiments which were done in triplicate. #$P<0.05$.

3.2. Involvement of EGFR in LPA-induced NF-κB activation

Because it is well known that LPA transactivates EGFR and this transactivation plays a vital role in LPA-induced MAPK activation process [4,23], we examined whether this is true in the case of the NF-κB activation. Tyrphostin AG1478, a rather selective EGFR-autophosphorylation tyrosine kinase activity inhibitor, or tyrphostin A25 treatment suppressed the NF-κB activation (Fig. 2A). In consistency

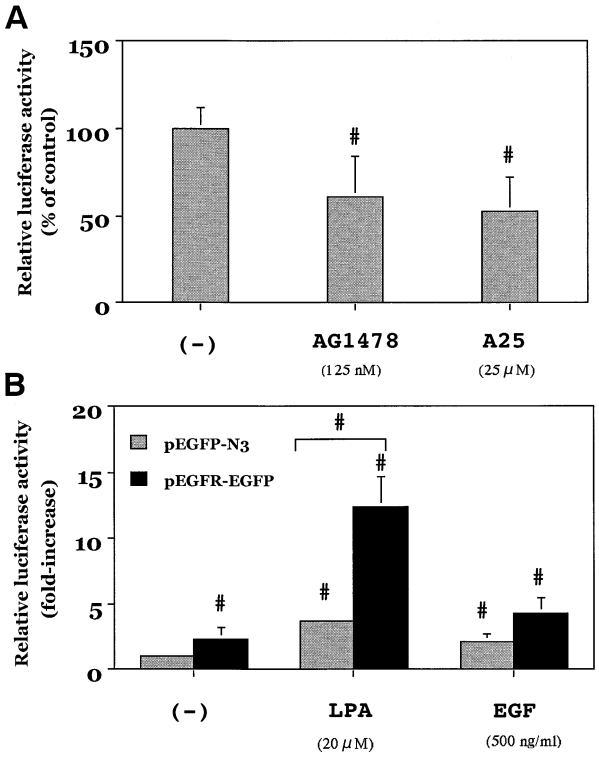

Fig. 2. Involvement of EGFR activation in LPA-induced NF-κB activation. NG108-15 cells were transfected with 250 ng of pNF-κB-Luc and 100 ng of pSV-β-galactosidase with (B) or without (A) 250 ng of pEGFR-EGFP or pEGFP-N3. 12 h after transfection, cells were treated with or without AG1478 (125 nM) or A25 (25 μM) for 1 h (A) and then treated with LPA. B: Cells were treated with LPA (20 μM) or EGF (500 ng/ml). After 6 h treatment, cells were harvested and underwent the luciferase assay. The results are the means ± S.D. and presented as percent induction in luciferase activity over the lane with LPA treatment (A) or as fold increase over the baseline seen with the pEGFP-N3-transfectant without treatment (B). This is a representative of two independent experiments which were done in triplicate. $\#P < 0.05$.

with this results, overexpression of EGF receptor significantly enhanced LPA-induced NF-κB-dependent luciferase activity compared to the pEGFP-N3 transfectants in NG108-15 (Fig. 2B). Treatment with 500 ng/ml of EGF stimulated NF-κB activation less than with 20 μM of LPA. AG1478 treatment abolished the enhancement of NF-κB activation by overexpression of EGFR in NG108-15 cells (data not shown).

3.3. LPA-induced EGFR transactivation is under redox regulation

Next we examined if LPA-induced EGFR activation is redox-sensitive or not. NG108-15 cells were transfected with pEGFR-EGFP and treated with LPA with or without exogenous TRX expression or NAC treatment. As shown in Fig. 3, LPA transactivated EGFR (lane 2) and this was suppressed by wild-type TRX expression (lane 3) or NAC treatment (lane 4).

3.4. Activation of NF-κB by Gq, G12 or G13 is TRX-sensitive

The LPA receptor has been shown to be a seven-transmembrane domain motif characteristic of GPCRs that is coupled to Gi, Gq, G12, or G13 subfamilies of heterotrimeric G protein [1]. LPA-induced NF-κB activation was suppressed by

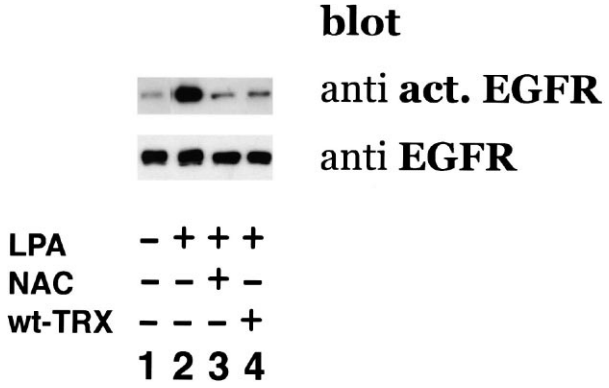

Fig. 3. Redox regulation of EGFR transactivation by LPA. NG108-15 cells were transfected with pEGFR-EGFP with pcDNA3 or pcDNA3-TRX-wt. After 12 h, cells were serum-starved for 12 h and then stimulated for 15 min in the absence or presence of LAP (40 μM). Cells were harvested and then subjected to Western blotting analysis as described in Section 2.

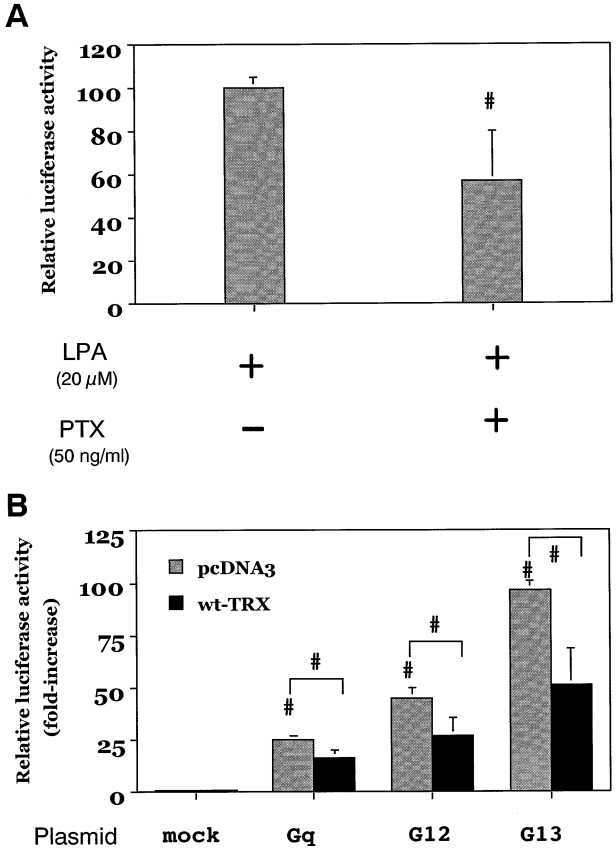

Fig. 4. Activation of NF-κB by Gq, G12 or G13 is TRX-sensitive. NG108-15 cells were transfected with 250 ng of pNF-κB-Luc and 100 ng of pSV-β-galactosidase. 6 h after transfection, cells were treated with 50 ng/ml of PTX for 18 h and then stimulated with LPA for 6 h (A). B: Cells were transfected with 250 ng of pNF-κB-Luc, 100 ng of pSV-β-galactosidase, and 250 ng of pcDNA3-Gq Q209L, pcDNA3-G12 Q229L, or pcDNA3-G13 Q226L with 250 ng of pcDNA3 or pcDNA3-TRX-wt. Cells were incubated for 18 h. The results are the means ± S.D. and presented as percent induction over the lane without PTX treatment (A) or as fold increase over the mock transfectant (B). This is a representative of two independent experiments which were done in triplicate. $\#P < 0.05$.

PTX treatment (Fig. 4A). This suggests that part of the NF-κB-stimulating action of LPA is assigned to Gi. As next, we tried constitutively activated form of Gq, G12, and G13. As shown in Fig. 4B, overexpression of constitutively activated G proteins by itself stimulated NF-κB activation. These activations were inhibited by overexpression of TRX or IκBα32A/36A (data not shown).

3.5. Involvement of small GTPase in LPA-induced NF-κB activation

Shahrestanifar et al. have reported that LPA stimulates generation of ROI in HeLa cells and activation of NF-κB [5]. This process is reported to be sensitive to DPI, a potent inhibitor of flavonoid proteins, such as NADPH oxidase [24]. We investigated a possibility of involvement of a Rho family small G protein, Rac1, which is one of the essential components of NADPH oxidase system in even non-phagocytotic cells [24]. As shown in Fig. 5A, overexpression of dominant negative mutant of Rac1, Rac1-N17 dose-dependently suppressed LPA-induced NF-κB activation. Moreover, overexpression of constitutively activated Rac1 mutant, Rac1-V12 by itself stimulated NF-κB activation in NG108-15 cells as well as HEK293 cells and this was also TRX-sensitive (Fig. 5B). Expression of dominant negative form of Rho also suppressed NF-κB activation as reported in HeLa cells [25].

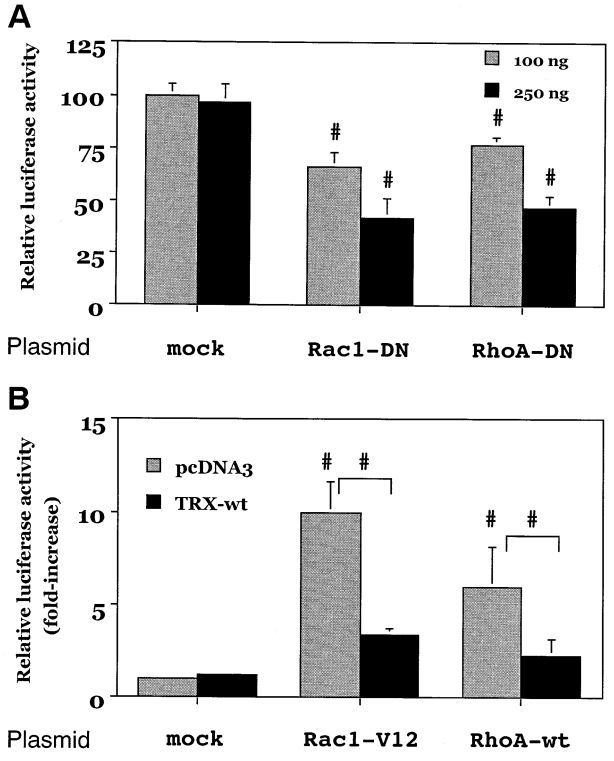

Fig. 5. Involvement of small GTPase in LPA-induced NF-κB activation. NG108-15 cells were transfected with Rac1-DN or RhoA-DN expression plasmids with 250 ng of pNF-κB-Luc and 100 ng of pSV-β-galactosidase. At 12 h after transfection, cells were treated by LPA. After 6 h incubation, cells underwent the luciferase assay (A). B: Cells were transfected with 250 ng of Rac1-V12 or RhoA-wt expression plasmids with 250 ng of pNF-κB-Luc and 100 ng of pSV-β-galactosidase with pcDNA3 or pcDNA3-TRX-wt. After 24 h, cells were harvested and subjected to luciferase assay. The results are the means ± S.D. and presented as percent induction over the mock transfectant (A) or as fold increase over the mock transfectant (B). This is a representative of two independent experiments which were done in triplicate. $\%P < 0.05$ compared with pEGFP-N3/TNF, $\#P < 0.05$ compared with pEGFR-EGFP/TNF.

4. Discussion

In this study, we have shown that LPA stimulates NF-κB-dependent transcription in a redox-sensitive manner in NG108-15 cells. The dose of LPA required for NF-κB activation was 10 μM in this study. This is higher than that reported to inhibit adenylyl cyclase, activate ERKs, elevate IP3 and Ca^{2+}, and stimulate formation of stress fiber and focal adhesions but is similar to that needed for activation of serum response factor and reinitiation of DNA synthesis [3].

The molecular mechanism of LPA-induced NF-κB activation is largely unknown. Recently, in addition to TNF-receptor-RIP-TRAF-NIK pathway, an alternative signalling pathway has been described in certain cells in which Akt directly phosphorylates IKK leading to the activation of NF-κB, independent of MAPK/ERK kinase kinase-1 (MEKK1) and NF-κB inducible kinase (NIK) [26,27]. This pathway thus allows growth factors such as PDGF to activate NF-κB [28]. Laffargue et al. clearly demonstrated that LPA induces phosphoinositide 3-kinase activation in a EGFR-dependent manner [29]. We also have demonstrated that two different PI3K inhibitors and EGFR inhibitors suppress NF-κB-dependent gene expression. Another possible mechanism is the MEKK1-dependent pathway. MEKK1 is another MAPK kinase kinase which induces the degradation of IκBα and activates NF-κB-dependent reporter gene activation [30]. However, we have demonstrated that NF-κB activation by overexpression of activated MEKK-1 or IKKα is TRX-sensitive in neither HEK293 cells [22] nor NG108-15 cells (data not shown). ROIs enhance the EGFR phosphorylation in ligand-independent manners [31] and H_2O_2 treatment induced EGFR phosphorylation in NG108-15 cells and A431 cells (K.H., unpublished observation). This is consistent with our results with NAC and TRX (Fig. 3). Although it is unclear how ROI is involved in this process, a plausible mechanism is the inactivation of protein tyrosine phosphatases (PTP) [32]. Because all PTPs have catalytic sites containing reactive cysteine residues, which form a thiol-phosphatase intermediate during catalysis, oxidation of these residues leads to their inactivation and overexpression of thiol-reducing TRX may reduce oxidized residues.

The LPA receptor is a GPCR that is coupled to Gi, Gq, and G12/13 subfamilies of G proteins [1]. Our results using PTX show that Gi is involved in this process, although we do not have any definitive evidence to which G proteins are coupled to LPA receptor and play a major role in LPA-induced NF-κB activation in NG108-15 cells. However, evidence that the activation by LPA is not completely suppressed by 50 ng/ml of PTX suggests that all of the action by LPA is not assigned to Gi (Fig. 4A). Actually, Gq is expressed in NG108-15 cells, and overexpression of constitutively active Gq stimulates NF-κB activation (Fig. 4B). Overexpression of constitutively active G12 or G13 stimulates NF-κB activation in NG108-15 cells (Fig. 4B), although involvement of this G12/13 family G protein is to be investigated more as further study. One of the downstream targets of both Gi and Gq is the phosphoinositide-specific phospholipase C-β, whose activation results in recruitment and activation of PKC [33].

Shahrestanifar et al. demonstrated that in Swiss 3T3 cells, a PKC inhibitor, Ro-31-8220, inhibits NF-κB activation by LPA [5]. PKC activation has been shown to be under redox regulation. H_2O_2 treatment stimulates PKC activation and treatment with NAC or another antioxidant suppresses PKC activation by extracellular stimuli [34]. Interestingly, TRX has been identified as one of the PKC-binding proteins and recombinant TRX inhibits PKC phosphorylation activity in an in vitro experiment [35]. Another common target for Gi, Gq, G12, and G13, is the Rho family small GTPase protein. Rho has been demonstrated to be a downstream molecule of LPA [36]. Our results strongly suggest that in NG108-15 cells in addition to Rho, another type of small G protein, Rac1, plays an significant role in the LPA-stimulated NF-κB activation pathway. Rac1 is an essential subunits of NADPH oxidase system in even non-phagocytotic cells [24] and overexpression of constitutively active mutant of Rac1 confers generation of ROI in certain cells [24,37] and overexpression of dominant negative mutant suppresses ROI generation by cytokines such as TNF and IL-1 [38], and growth factors such as EGF [39] and PDGF [40,41]. Because TRX has a peroxidase activity like catalase by itself [16] and is an electron donor to peroxidase such as peroxiredoxin (Prx) [42], TRX scavenges H_2O_2, one of the second messengers in the intracellular signalling pathway.

LPA regulates life and death of various types of cells. NF-κB is also shown to play a critical role in regulating cell fate [43]. Recently a chemokine, fractalkine, produced by glia cells activates PI3K and NF-κB via the CX(3)CR1 receptor, which is a GPCR and puts a survival signal into neurons [44]. It seems very interesting to examine the involvement of EGFR in this system.

In summary, we have demonstrated that an endogenous redox-acting molecule, TRX, regulates LPA-induced NF-κB activation at various levels of signalling pathways in NG108-15 cells.

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