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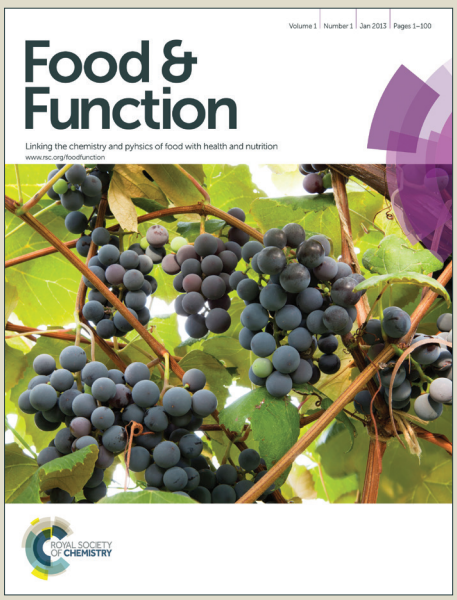

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Attenuated migration by green tea extract (-)-epigallocatechin gallate (EGCG): involvement of 67 KDa laminin receptor internalization in macrophagic cells

Xuezhi Ren[a,b], Xingzhi Guo[a,b], Li Chen[a], Minxia Guo[a], Ning Peng[a], Rui Li[a,*],

[a]Department of Neurology, Shaanxi Provincial People's Hospital, Xi'an 710068, China.

[b]The Third Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an 710061, China.

*Correspondence should be addressed to Dr. Rui Li, Department of Neurology, Shaanxi Provincial People's Hospital, No. 256, Youyi West Road, Xi'an 710068, China. Tel: +86 13096923029.

E-mail address: lrmail@yeah.net

Abstract

Excessive microglia activation in the brain is involved in the process of neurodegenerative diseases. Previous study indicated that (-)-epigallocatechin gallate (EGCG), a major active constituent of green tea, exhibited potent suppression on microglia activation. Since 67 KDa laminin receptor (67LR) is a key element in the cellular activation and migration, we investigated the effect of EGCG on cell migration and 67LR in lipopolysaccharide (LPS)-activated macrophagic RAW264.7 cells. Presence of EGCG (1-25 μM) markedly attenuated LPS-induced cell migration in a dose-dependent manner. Meanwhile, total 67LR protein in the RAW264.7 cells was unaffected by EGCG as revealed by Western-blot analysis. Further, confocal immunofluorescence indicated that EGCG caused a marked membrane translocation of 67LR from membrane side towards cytoplasm. Cell surface biotinylation analysis confirmed that EGCG dose-dependently induced a significant internalization of 67LR by 24%~68%. The study helps the understanding of pharmacologic action of EGCG on 67LR, implicating the potential use in the macrophage/microglia activation-associated illnesses such as neurodegenerative diseases and cancers.

Keywords

Neurodegenerative disease; macrophage; (-)-epigallocatechin gallate; 67 KDa laminin receptor

Introduction

Microglial cell is the innate macrophagic population in the central nervous system, maintaining the microenvironmental homeostasis.[1] Subtle activation of microglia is crucial to neuronal function and survival, however, excessive activation of microglia is regarded to be detrimental to neuronal health.[2] Activated microglia switches its phenotype from a resting status to activated one when certain stimuli exists, exhibiting amoeboid shape, secretion of proinflammatory factors and ability of migration and phagocytosis.[3] Accumulating data have revealed that chronic activation of microglia is potentially involved in the process of various illnesses, especially neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[4, 5] Thus, it was widely accepted that blockade of microglial activation could be a potential therapy for neurodegenerative diseases.[6, 7]

Consumption of green tea is supposed to be linked with the protection of cancer, neurodegenerative diseases and cardiovascular diseases.[8] We previously found that green tea extract (-)-epigallocatechin gallate (EGCG) could markedly suppress the activation of microglia, with a significant reduction in the consequent release of proinflammatory mediators, such as tumor necrosis factor-α (TNFα) and nitric oxide.[9] More recent study showed that EGCG blocked downstream pathways to prevent the expression of pro-inflammatory gene in macrophages.[10]

Additionally, EGCG could profoundly affect the activated microglia, showing its neuroprotective properties both *in vivo* and *in vitro*.[11] However, the mechanism by which EGCG exerts its pharmacological effects against macrophage/microglia still remains to be elucidated.

The 67KDa laminin receptor (67LR), isolated and identified from tumor cells, is primarily a crucial element in cancer invasion and metastasis.[12, 13] Recently, it has been described that 67LR is not only highly expressed by microglia, but also essential to activation and neurotoxicity of microglia.[14] In addition, microglia phenotypes and functions are closely related to the interaction between adhesion molecules and its extracellular matrix. For example, α6β1 integrin binding to laminin is an essential prerequisite for microglial activation.[15] Furthermore, the functions of microglia to recognize and phagocytize foreign materials as amyloid β plaques were mediated by cell surface receptor complex including 67LR.[16] Significantly, recent studies have identified 67LR as a receptor of EGCG on the cell surface with highly specific affinity.[17] In macrophage cells, EGCG could regulate the Tollip-TLR4 signaling pathway through 67LR to reduce subsequent inflammatory processes.[18] Thus, these data suggest that 67LR is a key molecule involving in macrophage/microglia activation, and a putative mediator for the inhibitory effects of EGCG.

We have previously identified that 67LR is expressed both in primary microglia and a macrophage cell line RAW264.7 which resembles the

most properties of microglia biologically and pharmacologically. [1] In this study, we investigated the effects of EGCG on cell migration and 67LR in the RAW264.7 cells to further understand the molecular mechanism underlying the activities of EGCG.

Experimental methods

Materials

Mouse RAW264.7 macrophage cells were obtained from Shanghai Institute of Cells, Chinese Academy of Sciences (Shanghai, China). Transwell system was purchased from Costar, Corning, NY (USA). LPS (Escherichia coli 0111:B4) and EGCG (>95% in purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-67LR monoclonal antibody (MLuC5) was purchased from Abcam (USA). Anti-β-actin mouse monoclonal antibody was obtained from Cowin Biotech Co. Ltd (Beijing, China). EZ-Link Sulfo-NHS-SS-Biotin and biotinylation kits and enhanced chemiluminescence's (ECL) reagents were purchased from Thermo Fisher Scientific (USA). Horseradish peroxidase-conjugated anti-mouse antibody and FITC-conjugated antibody to 67LR were purchased from Bioss Biotech Co. Ltd (Beijing, China). Dulbecco's modified Eagle medium (DMEM) and heat-inactivated endotoxin-free fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA).

Cell migration analysis

To investigate the effect of EGCG on cell migration, RAW264.7 cells (5.4 $\times 10^4$/insert) were placed onto Matrigel-coated Transwell inserts with 8-μm pore size, priorly equilibrated by incubation in DMEM (high glucose) supplemented with 2% FBS for 2 h at 37°C. Afterwards, the upper chamber was inserted into a 24-well plate containing 800 μl DMEM medium supplemented with or without LPS (500 ng/ml) and indicated final concentration of EGCG. Migration was measured after incubation at 37°C and 5% CO_2 for 5 h. Cells attached on the upper side of the transwell membrane were removed and cells on the lower surface were fixed and visualized using the crystal violet staining. Stained membranes were digitally imaged, and the number of cells was counted at four randomized locations on each membrane by an investigator blind to the experimental design. The numbers of cells treated with LPS alone were used as control.

Cell culture and treatment

RAW264.7 macrophage cells were grown to confluence by 80-90% in DMEM (high glucose) supplemented with 10% heat-inactivated FBS and 1% penicillin (50 U/ml)/streptomycin (50 μg/ml) under a humidified condition of 37°C, 5% CO_2. After being gently washed with PBS and cultured in serum-free DMEM, the cells (1×10^5 cells/ml) were treated

with various concentrations of EGCG (1-25 μM) for 1 h and then LPS (500 ng/ml) for 6 h.

Western blot analysis for total cellular 67LR

To determine the effects of EGCG on 67LR protein, Western blot analysis was performed to semiquantitative detection of 67LR. The cells with indicated treatment were washed with 0.1 mM phosphate-buffered saline (PBS) and lysed with radio immunoprecipitation assay (RIPA) buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The total protein level was quantified with a BCA protein assay kit. Each sample (50 μg) was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes and incubated with 5% skim milk for 1 h at room temperature to block nonspecific protein binding, followed by incubation with the anti-67LR antibody (1:1000) overnight at 4°C. After being washed completely with PBS containing 0.1% Tween-20, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody for 2 h at room temperature and detected by enhanced chemiluminescence (ECL) reagents. Detection of β-actin was served as an internal control.

Immunofluorescence assay for cellular distribution of 67LR

To detect the cellular location of 67LR, RAW264.7 cells were planted on the coverslips in 6-well plates (5×10^5 cell/well), and treated with LPS and

then different concentrations of EGCG for 6 h as described previously. For immunofluorescence, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized with 0.3% TritonX-100 in PBS for 5 min at room temperature. After being washed three times with PBS, the cells were incubated with 5% BSA in PBS for 1 h at room temperature to block nonspecific binding. Mouse anti-67LR monoclonal antibody was used for immunostaining at 1:500 (diluted in 1% BSA). After being incubated overnight at 4°C, cells were washed again and then incubated with FITC-conjugated secondary antibody in the coverslips for 2 h at room temperature in dark, followed by counterstaining with diamidino-2-phenylindole (DAPI) for 5 min. After wash with PBS for 3 times, the cells were observed under a confocal laser microscopy (Olympus FV1000).

Surface and intracellular 67LR detection

RAW264.7 cells treated with various concentration of EGCG (1-25 μM for 6 h) were washed with PBS/Ca-Mg (phosphatebuffered saline with 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.3), before treatment with 1.5 mg/ml sulfo-NHS-SS-biotin for 40 min at 4°C in PBS/Ca-Mg to biotinylate cell surface 67LR. After being washed again with PBS/Ca-Mg containing 100 mM glycine, the cells were collected and lysed in RIPA buffer containing 1.0 mM PMSF and 1.0 mg/ml protease inhibitors cocktail. Afterwards, protein concentrations were determined and equal

amounts of cellular proteins (50 μg) were incubated with sulfo-NHS-SS-biotin for 45 min at room temperature with moderate agitation, as indicated in the instruction of EZ-Link biotinylation kits. Non-biotinylated proteins were precipitated with 10% TCA and resuspended in laemmli sample buffer. Biotinylated proteins were eluted with laemmli sample buffer, followed by being washed with RIPA buffer for three times. Western blot was performed with primary antibody to anti-67LR to detect biotinylated and nonbiotinylated proteins respectively.

Statistical analysis

The statistical analyses were conducted using SPSS 10.04 software (SPSS Inc.). All data were presented as mean ± SD. The significance of differences between groups was estimated by one-way ANOVA followed by *post hoc* comparison using Bonferonni's method. $P<0.05$ was considered to be statistically significant.

Results

Inhibited migration of RAW264.7 cells by EGCG

Cell migration analysis showed that RAW 264.7 cells exhibited a conserved migration without LPS stimulation. Administration of LPS induced a marked increase (about 3.5 fold over pretreatment) of migrated cell. Compared with LPS treatment, EGCG significantly inhibited the

LPS-induced cell migration by in a dose-dependent manner (Fig. 1).

Unaffected total cellular 67LR by EGCG

67LR has been shown to be involved in the regulation of anti-inflammation for EGCG. To further evaluate the effect of EGCG on 67LR, LPS-activated RAW264.7 cells were treated with different doses of EGCG (1, 5, 25 μM). Western blot analysis indicated that the treatment of EGCG did not alter the level of total 67LR in RAW264.7 cells (Fig. 2).

Confocal microscopy findings of EGCG-enhanced 67LR internalization

To observe the cellular localization in RAW264.7 cells, 67LR was visualized by confocal laser microscopy. The intensity analysis shows that total expression of 67LR was not altered in the cell, regardless of the dose of EGCG, which was in consistent with Western blot analysis indicated above. In contrast, LPS challenge induced a marked redistribution of 67LR in the cells. 67LR in resting RAW264.7 cells expressed predominately located in the cellular compartment, while stimulation of LPS for 6 h induced an eggshell-like distribution of 67LR (white arrow), indicating the increased surface 67LR. Meanwhile, presence of EGCG exerted a reversed distribution of 67LR in the RAW264.7 cells, namely, the reduction of surface expression and an increasing in the intracellular expression, in parallel with a phenotype

switching into a resting status. The results indicated that EGCG could regulate an internalization of 67LR in LPS-induced RAW264.7 cells (Fig. 3).

Surface biotinylation of 67LR in RAW264.7 cells

Internalization，also known as membrane traffic from surface to intracellular compartment, characterized as receptor-independent endocytosis, is an important mechanism for the regulation of surface large molecules.[19] To confirm the effects of EGCG on the 67LR of RAW264.7 cells， we investigated the effects of EGCG on surface and intracellular 67LR distribution by biotinylation of cell-surface proteins. The quantitative results revealed that more than 65% of total 67LR located in the intracellular compartment of cultured RAW264.7 cells, while 35% of total 67LR located on the cell surface. LPS-stimulation significantly altered the distribution of 67LR, with a dramatic increase (over 40%) of 67LR on the surface. EGCG (1-25 μM) could reverse the effects of LPS and increase the expression of 67LR in the cytoplasm by 24%, 57%, 68%, respectively (Fig. 4).

4. Discussion

EGCG is a major component of green tea polyphenols with multiple bioactivities, can potentially suppress the activation of microglia to attenuate inflammation responses.[9, 20] EGCG has also been found to

inhibit metastasis in various types of cancer.[21-23] To further elucidate the possible mechanism for the inhibition of microglia activation and tumor metastasis, we investigated the effects of EGCG on cell migration and 67LR in macrophagic cells. It indicated that EGCG was capable of inhibiting cell migration and regulating LPS-enhanced membrane trafficking of 67LR in RAW264.7 cells, suggesting that the internalization of 67LR may serve as a mechanism underlying the inhibitory effects of EGCG on microglia activation and migration. To our knowledge, it is the first demonstration of a novel pharmacologic action of EGCG.

67LR is a high affinity cell-surface receptor for its ligand laminin, which is rich in cellular matrix, and acts as an important molecule both in cell adhesion to migration.[24] Over-expression of 67LR was a common profile shared by macrophage/microglia and various types of cancer, suggesting its critical roles in the metastasis and migration of the cells.[24, 25] In fact, laminin receptor precursor has been found to be closely related microglia/macrophage activation both in vivo and in vitro.[5] Blocking of 67LR with its inhibitor resulted in a suppression of microglia activation. Then, it was proposed that 67LR may be a potential target to the treatment of cancer or microglia/macrophage activation-related illnesses.

It is well known that internalization or endocytosis is an important regulatory mechanism not only for the process of extracellular substances

but the function of most cell-surface receptors.[19] For example, 67LR-dependent binding and endocytosis are contributed to the absorption of bovine prions.[26] We previously demonstrated that EGCG can regulate dopamine transporter (DAT) internalization through protein kinase C pathway in DAT-overexpressed PC12 cells,[27] which is believed to be associated with its capability of neuroprotection. In the study, we addressed that the cell surface of 67LR in macrophagic cells was enhanced by LPS stimulation, while EGCG reversed the effect of LPS, inducing a marked internalization of surface 67LR. Theoretically, internalization of 67LR may result in a reduction of 67LR-associated functions of cell motility, migration and invasion. Furthermore, EGCG can attenuate the expression of inflammatory cytokines by blocking MAPK and NF-κB signal-transduction pathways, which is dependent on 67LR in macrophage cells.[18] In addition, the extracellular 67LR-binding domain of EGCG has been identified as extracellular domain (residues 161-170).[28] Moreover, peripherally administrated EGCG is capable of penetrating into cerebral tissues, although in a limited proportion, implicating the potential application of EGCG in the central nervous system diseases.[29] Thus, 67LR internalization-targeted molecular approaches would be potentially contributed to the treatment of several kinds of diseases, especially cancers and neurodegenerative diseases.

5. Conclusion

In summary, our study suggests that EGCG promotes 67LR internalization and attenuates cell migration in activated macrophagic cells. Considering the vital role of 67LR in cellular migration and motility, particularly in macrophagic cells, these findings help the understanding of pharmacologic properties of EGCG. It also implicates the potential use in the macrophage/microglia activation-associated illnesses such as neurodegenerative diseases and cancers.

Acknowledgments

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Figures Legends

Fig. 1 LPS and EGCG effects on cell migration. A transwell insert with 8-μm pore size was used to investigate cell migration. RAW264.7 cells (5.4×10^4/insert) were incubated in DMEM with 2% FBS and then inserted into a 24-well plate containing DMEM medium supplemented with or without LPS (500 ng/ml) and indicated final concentration of EGCG. The numbers of migrated cells treated with DMEM (vehicle) were defined as control (100%). As compared with the control, LPS (500 ng/ml) induced a marked increase of migrated cell number. EGCG significantly inhibited the LPS-induced cell migration in a dose-dependent manner, as compared with LPS treatment. *P<0.01 compared with the control group, and #P<0.01 compared with LPS group.

Fig. 2 Effects of EGCG on the expression of total 67LR in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with EGCG (1, 5, 25 μM) for 1 h and stimulated with or without LPS (500 ng/ml) for 6 h. The total 67LR was detected by Western blotting (A). β-actin in equal amount of protein were severed as internal control. The protein band density was scanned and quantified with Image J (B). The assay was performed three times, from which all data are expressed as mean ± SD.

Fig. 3 Changes in spatial distribution of 67LR after treated with EGCG in LPS-stimulated RAW264.7 cells. (A) Immunofluorescence staining was performed with anti-67LR antibody, followed by FITC-conjugated secondary antibody (green). The nucleuses were redyed with DAPI (blue). 67LR was predominantly located in the intracellular compartment in resting RAW264.7 cells (top row of panel A, as indicated with white arrow). After stimulated with LPS, 67LR appeared to be concentrated to the cell surface, giving an eggshell-like appearance (the second row of panel A). However, pretreatment with EGCG (1-25 μM) reversed the effects of LPS in 67LR distribution in a dose dependent manner. (B) The mean fluorescence intensity of 67LR was quantified with Image J. All values are obtained and expressed as mean ± SD from three separate experiments. Scale bar=10 μm.

Fig. 4 Effects of EGCG on surface and intracellular 67LR in LPS-stimulated RAW264.7 cells.

RAW264.7 cells treated with various concentration of EGCG (1-25 μM for 6 h) prior to LPS challenge. Surface biotinylation method with sulfo-NHS-SS-biotin was used to distinguish the surface and intracellular protein. Localization of surface and intracellular 67LR was determined by Western blotting, and the above images were representative of three experiments (A). All data, obtained by Image J software, were expressed as mean ± SD. *$P<0.05$ compared with the control group and #$P<0.05$ compared with LPS-stimulated group respectively.

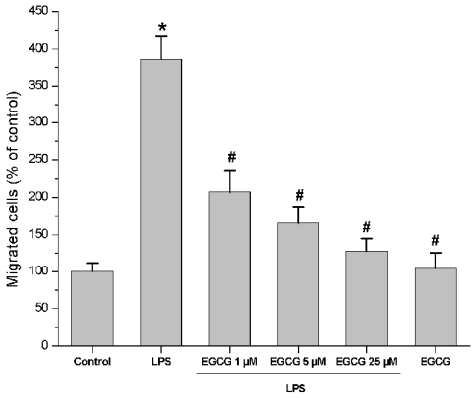

Figure 1

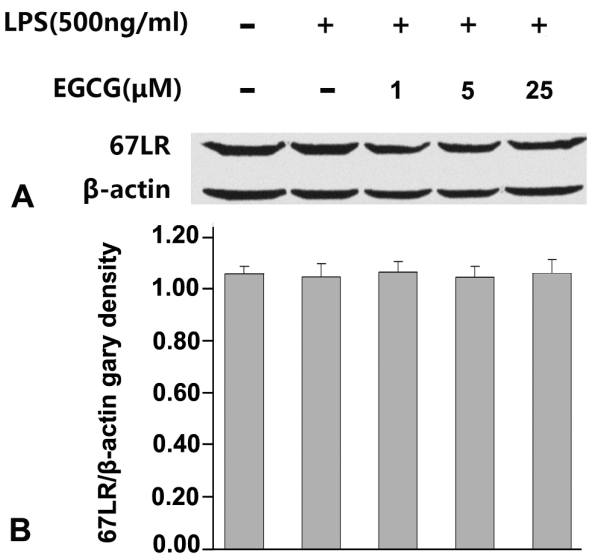

Figure 2

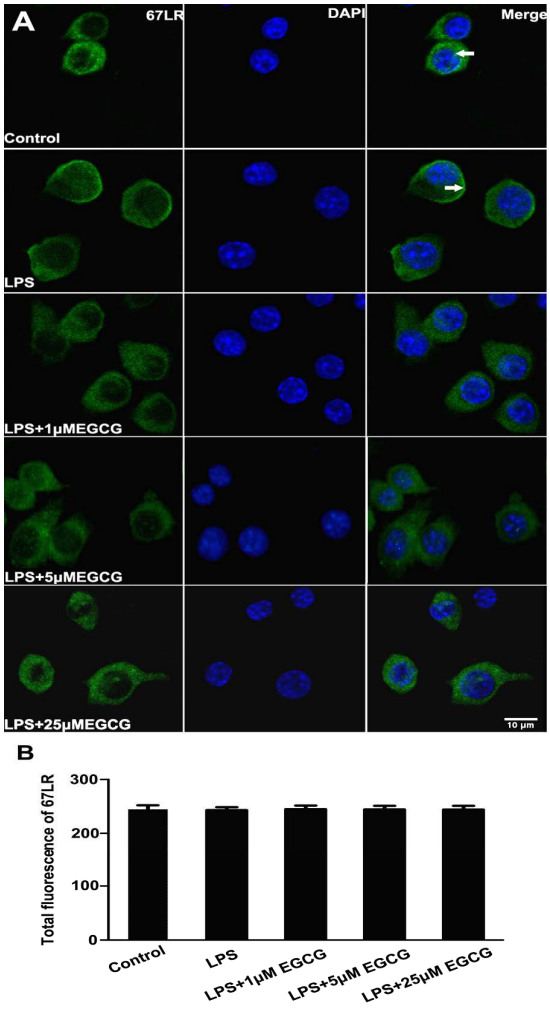

Figure 3

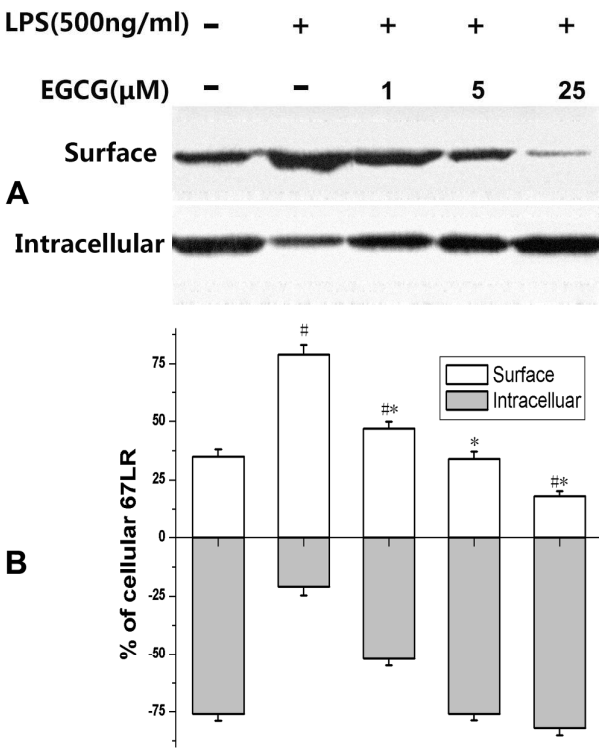

Figure 4