Title: Anti-inflammatory role of Leptin in glial cells through p38 MAPK pathway inhibition

Author: Iván Patraca Nohora Martínez Oriol Busquets Aleix Martí Ignacio Pedrós Carlos Beas-Zarate Miguel Marin Miren Ettcheto Francesc Sureda Carme Auladell Antoni Camins Jaume Folch

PII: S1734-1140(16)30427-3
DOI: http://dx.doi.org/doi:10.1016/j.pharep.2016.12.005
Reference: PHAREP 606

To appear in:

Received date: 28-7-2016
Revised date: 27-10-2016
Accepted date: 8-12-2016

Please cite this article as: Iván Patraca, Nohora Martínez, Oriol Busquets, Aleix Martí, Ignacio Pedrós, Carlos Beas-Zarate, Miguel Marin, Miren Ettcheto, Francesc Sureda, Carme Auladell, Antoni Camins, Jaume Folch, Anti-inflammatory role of Leptin in glial cells through p38 MAPK pathway inhibition, http://dx.doi.org/10.1016/j.pharep.2016.12.005

Highlights

Pro-inflammatory cytokines increase in inducible nitric oxide synthase in glial cells

Leptin produced an inhibitory effect on iNOS expression and NO production

Leptin decrease in p38 MAP Kinase (MAPK) pathway activity

Leptin reduced the activation of apoptosis though cytochrome c (Cyt-c) release and caspase-3 inhibition

Anti-inflammatory role of Leptin in glial cells through p38 MAPK pathway inhibition

Iván Patraca[1,3], Nohora Martínez[1,3], Oriol Busquets[1], Aleix Martí[1], Ignacio Pedrós[1,2], Carlos Beas-Zarate[5,6], Miguel Marin[7], Miren Ettcheto[2,3], Francesc Sureda[1,3], Carme Auladell[4], Antoni Camins[2,3,7] and Jaume Folch[1,3]

[1]*Unitats de Bioquímica i Farmacologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili*, C./ St. Llorenç 21 43201 Reus (Tarragona), Spain.

[2] *Unitat de Farmacologia i Farmacognòsia, Institut de Neurociencias, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain.*

[3]*Biomedical Research Networking Center in Neurodegenerative Diseases (CIBERNED), Madrid, Spain*

[4] *Departament de Biologia Cel·lular, Facultat de Biologia, Universitat de Barcelona*, Barcelona, Spain.

[5]Departamento de Biología Celular y Molecular, C.U.C.B.A., Universidad de Guadalajara, División de Neurociencias.

[6] Instituto Mexicano del Seguro Social (IMSS), Centro de Investigación Biomédica de Occidente (CIBO). Sierra Mojada 800, Col. Independencia, Guadalajara, Jalisco 44340, México.

[7] Centro de Biotecnología. Universidad Nacional de Loja, Av. Pío Jaramillo Alvarado y Reinaldo Espinosa, La Argelia. Loja. Ecuador.

RUNNING TITLE: Anti-inflammatory role of Leptin in glial cells.

Correspondence and reprint requests to: Antoni Camins PhD

Unitat de Farmacologia i Farmacognosia, Facultat de Farmàcia Universitat de Barcelona, Spain.

Avda/ Diagonal 643, E-08028 Barcelona, Spain.

Tel: +34 93 4024531, Fax: +34 934035982

Email: camins@ub.edu

KEYWORDS: Leptin, Glial cells, iNOS, p38 MAPK, Caspase-3, Cytochrome-c

Additional information:

Author Contributions

a) **Study design and interpretation of data:** Jaume Folch, Antoni Camins, Carlos Beas-Zarate, Miguel Marin, Francesc Sureda Carme Auladell.

b) **Writing of the manuscript**: Jaume Folch , Antoni Camins, Carlos Beas-Zarate, Miguel Marin, Francesc Sureda Carme Auladell.

c) **Study collection, analysis data and statistical analysis:** Iván Patraca, Nohora Martínez, Oriol Busquets, Aleix Martí, Ignacio Pedrós, Miren Ettcheto

All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This study was funded by grant 2009/SGR00853 from the Generalitat de Catalunya (autonomous government of Catalonia), by grants SAF2011-23631 from the Spanish Ministerio de Ciencia e Innovación. We also would like to express our gratitude to Ms Ana Nieto for revising this manuscript.

Anti-inflammatory role of Leptin in glial cells through p38 MAPK pathway inhibition

KEYWORDS: Leptin, Glial cells, iNOS, p38 MAPK, Caspase-3, Cytochrome-c

ABSTRACT

Background: In the present work, we studied the modulatory effect of Leptin (Lep) against pro-inflammatory cytokines, tumour necrosis factor-alpha (TNFα), interleukin 1-beta (IL1β) and interferon-gamma (IFNγ), in primary glial cell cultures.

Methods: Glial cultures were treated with pro-inflammatory cytokines (TNFα, 20 ng/ml; IL1β, 20 ng/ml; IFNγ 20 ng/ml). Cells were pre-treated with Lep 500nM, 1 hour prior to cytokine treatment. NO released from glial cells was determined using the Griess reaction. Cell viability was determined by the MTT method. Protein expression was determined by western blot.

Results: Pre-treatment with 500 nM Lep produced an inhibitory effect on inducible Nitric oxide synthase (iNOS) expression and nitric oxide (NO) production after glial cells exposure to pro-inflammatory cytokines. Anti-inflammatory effect can be related to a decrease in P38 MAP Kinase (MAPK) pathway activity. Treatment of glial cell cultures with Lep also reduced the intrinsic apoptotic pathway (cytochrome c release and caspase-3 activation).

Conclusions: We suggest that Lep would act as an anti-inflammatory factor in glial cells exposed to pro-inflammatory cytokines, exerting its function on p38 MAPK pathway and reducing NO production.

Introduction

Preclinical and clinical studies have demonstrated a crucial role of glial cells, mainly the microglia activation in the increased synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β, IFNγ and IL-6 associated with brain damage [1,2]. Thereby a neuroinflammatory process may enhance the risk of onset of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [3-6]. In addition, chronic inflammatory processes are common in obesity and cancer. Moreover, glial cells mainly astrocytes exert important functions in the brain, among them synthesis of neurotrophic factors, ion homeostasis, and regulation of extracellular glutamate concentration [7,10]. Likewise, overactivation of both astrocytes and microglia favor neurotoxic factors as nitric oxide, reactive oxygen species, and glutamate increase [11-15]. The excessive levels of nitric oxide are harmful for neurons and induce protein alterations by means of nitrosylation and nitration. This is probably one of the main mediators of neuronal cell loss [12]. Accordingly, activation of iNOS in glial cells constitutes a critical event in inflammatory-mediated neurodegenerative disorders, mainly as a result of microglial activity [11,12,14]. As we discussed above, pro-inflammatory cytokines, will be the main brain responsible mediators for inducible iNOS activation in glial cells [16,19].

The adipokine leptin is synthesized mainly in the adipose tissue and circulating Lep cross the Brain Blood Barrier (BBB) into the brain, where it exerts key functions in the physiological regulation of food intake, glucose homeostasis, and energy expenditure via the hypothalamic circuits regulation [20-22]. However, functional Lep receptors (LepR or ObRb) are located in the hippocampus and cortical regions of the brain [23-25]. Thus, Lep receptors can be related to hippocampal learning and memory, including activity-dependent synaptic plasticity and the trafficking of glutamate receptors to-, and away from- the hippocampal synapses [25,26]. Furthermore, impairments in Lep function could induce apoptosis among hippocampal and it appears

that Lep confers protection against hippocampal neurodegeneration observed in AD disease [17]. Previous studies reported that Lep has also neuroprotective actions in AD through the inhibition of BACE1 and GSK3β and affects memory processes [24]. In addition Lep is involved systemic inflammation which is associated with obesity [21,22,23]. Besides, it has been reported that chronic inflammation is known to cause Lep resistance that is a status related to type-2 diabetes mellitus and AD [20, 21].

So far, it exist a lack of information concerning the evaluation of Lep role in the control of inflammatory activity by glial cells. Thus, the aim of the present work was to assess the anti-inflammatory potential effects of Lep, in mixed glial cell cultures treated simultaneously with three pro-inflammatory cytokines TNFα, IL1β and IFNγ.

MATERIALS AND METHODS

Animals and in vitro cell culture procedure

Three-day-old pups of C57BL/6 mice (Harlan, IN, USA) were used in this study. All animal care and experimental protocols with post-natal pups were carried out in accordance with the Directive 86/609EEC of the Council of the European Union. Every effort was made to minimize animal suffering and reduce the number of animals used.

Mixed glial cell cultures where prepared from the cerebral cortices from 3-day-old C57BL/6 mice, and according with Deumens et al., (2004) [27]. Briefly, cerebral cortices where dissected and meninges removed. The tissues were incubated with trypsin solution (Trypsin 0.05% (w/v) trypsin-EDTA 5mM) for 15 min at 37ºC. After that, the tissues were fragmented with a fire-polished Pasteur pipette. The dissociated cells were plated into 60 mm diameter culture plates (300,000 cells/ml) and grown in Dulbecco's Modified Eagle's /F-12 medium, supplemented with 10% fetal bovine serum, 2% HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin. Fresh medium was

changed the next day and then every 3 days afterwards. All experiments were done 7-9 days after plating.

Cell treatment

Glial cultures were treated with pro-inflammatory cytokines (CYT) (TNFα, 20 ng/ml; IL1β, 20 ng/ml; IFNγ 20 ng/ml) (PeproTech Inc., Rocky Hill, NJ, USA) or with the same volume of phosphate buffer saline (PBS) as a control. As indicated, cells were pre-treated with Lep (500nM; Sigma, St. Louis, MO, USA), 1 hour prior to CYT treatment, or with Lep alone as a control. In addition, cells were pre-treated with either SB203580 kinase inhibitor (Calbiochem, EMD Millipore USA), and CYT at concentrations of 20ng/ml, and in order to test the specificity of Lep inhibitory effect on p38 MAPK pathway.

Cell viability

Cell viability assays were performed after 48 hours of treatment, and were determined using MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma, St. Louis, MO, USA] [28].

Immunofluorescence and Hoechst staining

Mixed glial cell culture was characterized using two specific glial markers GFAP (astrocytes) and Iba 1 (microglia) as described in previous papers of our group [28]. The presence of Ob receptor was determined by immunofluorescence with Ob-R (H-300) sc-8325 antibody (Santa Cruz Biotechnology). Slides were allowed to defrost at room temperature and then were rehydrated with Phosphate-buffered saline (PBS) for 5 min. Later, the brain sections were incubated with 0.3% Thioflavin S (Sigma-Aldrich) for 20 min at room temperature in the dark. Subsequently, these were submitted to

washes in 3-min series, specifically with 80% ethanol (2 washes), 90% ethanol (1 wash), and 3 washes with PBS. Finally, the slides were mounted using Fluoromount (EMS), allowed to dry overnight at room temperature in the dark, and stored at 4C.

For nuclei staining, cells were plated onto glass coverslips. After treatments, cells were fixed as described above, incubated for 10 min with Hoechst 33342 (1 µg/ml, Sigma, St. Louis, MO, USA) and then washed again. Coverslips were mounted with Fluoromont-G (Electron Microscopy Sciences, Hatfield, PA, USA), and conserved at 4ºC until observation in the fluorescent microscope (Olympus BX61). All viability results were expressed as percentages with respect to non-treated cells.

Nitric oxide measurement

NO released from glial cells was converted to nitrite in the culture medium, which can be determined using the Griess reaction [28].

Western-blot

After treating cells with cytokines and Lep, cells were washed twice with ice-cold phosphate-buffered saline and harvested in lysis buffer containing 150 mM NaCl, 5mM $MgCl_2$, 50mM Tris, 1mM EDTA, 1%Triton X-100, pH 7,2 with protease inhibitors (Complete, Roche Diagnostics, Germany). Protein concentration was determined using bicinchoninic acid protein assay with the Pierce BCA Protein Assay Kit (Pierce Company, Rockford, IL, USA). An average of 10 µg of protein was denatured at 95°C, for 5 min, in sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5%β-mercaptoethanol, 0.05% bromophenol blue) and samples were subjected to SDS–polyacrylamide gel electrophoresis (7.5% Acrilamide: N,N'methylbisacrylamide, 37,5:1: Sigma-Aldrich, St. Louis, MO USA) and subsequently transferred onto polyvinylidene fluoride membrane (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Hercules,

CA, USA). Membranes were incubated overnight at 4°C with primary antibodies against iNOS, monoclonal Anti-β-Tubulin and β-actin (1:20000; Sigma-Aldrich, St Louis, MO, USA), phospho-Stat3-Tyr705 (9131), phospho-p38 MAPK-Thr180/Tyr182 (4511), Cytochrome c Antibody, Cleaved Caspase-3 (Asp175), MnSOD2 (13141), Bcl-2 (50E3) and Bax (2772) antibody (all from Cell signalling) and Ob-R (H-300) sc-8325 (Santa Cruz Biotechnology). The blots were then washed thoroughly in TBS-T and incubated for 2 hours with a horseradish peroxidase-conjugated anti-IgG antibody, followed by the enhanced chemiluminescence detection system (Molecular Image Quantity ChemiDoc XRS+ BioRad) according to the supplier's instructions. Chemiluminescence measurements and semi-quantitative values were obtained using the corresponding software Imagelab (Bio-Rad) and the results were expressed as a percentage with respect to control. The corresponding β-actin was taken for each sample to normalize for any differences in gel loading.

Determination of P38 activity

The P38 activity assay was performed using the P38 MAP Kinase Assay Kit (Cell Signalling) following the manufacturer's recommendations. Briefly, P38 was immunoprecipitated from 200 µg protein. After that, the samples were washed and centrifuged twice in lysis buffer and kinase buffer. The supernatants were discarded. The ATP protein fusion (ATF) and kinase buffer, were added to each sample, which was then incubated at 37ºC for 30 min. After that, samples followed the Western-blot protocol described previously. The primary antibody was pATF2 in a 1:1000 dilution. Chemiluminescence measurements and semi-quantitative values were obtained using the corresponding software Imagelab (Bio-Rad) and the results were expressed as a percentage with respect to control.

Statistical analysis

Results were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Statistical analyses were carried out using the version 15 of SPSS (SPSS Sciences, Chicago, Illinois) (GraphPad Prism 5, La Jolla, CA, USA). Statistical significance was defined as a p value less than differences with $p < 0.05$ (*); $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant. In order to simplify the interpretation of results, comparisons were done between CYT bar with respect to control. Further comparisons were performed among CYT bar with respect to Lep and the combination of Lep+CYT groups.

Results

Effect of cytokines on viability of mixed glial cell cultures

Mixed glial cell culture was characterized using two specific glial markers GFAP (astrocytes) and Iba 1 (microglia), as we described in previous studies [28]. The expression of Ob receptor in cell cultures was constant from the first day (data non shown), however our results revealed a significant reduction in ObR content up to 9 days after plating cells, all the experiments were performed within the first 72 hours (Figure 1).

To investigate whether cytokine-treated glial cells show an impaired mitochondrial function, we treated primary glial cells with three pro-inflammatory cytokines TNFα, IL1β, and IFNγ at concentrations of 20ng/ml, for 48h. Likewise, we evaluated the viability of cell culture under each different treatment by MTT and the results evidence a slight impairment in cell viability in cells exposed to cytokines but was not significant (Figure 2a). However, we observed a significantly increased fraction of apoptotic cells when stained with Hoechst 33342 (Figure 2b, c-e).

Leptin decreased Nitric Oxide production mediated by pro-inflammatory cytokines in mixed cultures of glial cells

To determine NO production, we measured nitrite released into the culture medium using the Griess reagent. Then, we proceed in according with this observation and experiments were done with all three cytokines together (TNFα, IL1β and IFNγ) at concentrations of 20 ng/ml. In fact, a significant increase in NO release was observed in glial cells exposed to cytokines, and when compared with controls (Fig. 3a), Exposure of cells to different concentrations of Lep (50, 100 and 500 nM), for 1h prior the cytokine treatment, reduced significantly and dose-response the NO production at 48h, as it is also shown in Figure 3a.

To support this diminution of NO, we also evaluated whether Lep pre-treatment is able to suppress iNOS content by Western-blot. As expected, cytokine treatment induced a marked increase in iNOS levels in glial cells, which was significantly reduced by pre-treatment with 500nM Lep (Fig. 3b). Taken together, our current data prove that Lep inhibits the inflammatory activation of glial cells with respect to iNOS induction and NO production.

Leptin decreased Nitric Oxide production is related to P38 MAP Kinase pathway in glial cells

Since previous investigations of our group suggest that this observation can be related to P38 MAPK pathway [28] we intended to shown the effect of Lep on P38 pathway. In fact, results from the present investigation show a significant increase on the content of the active form of p38 (phospho-P38 Thr180/Tyr182) as a result of the exposure to cytokines, that can be reversed by pre-treatment of glial cells with Lep (Figure 4a).

The present results show that Lep is able to inhibit specifically p38 MAPK pathway, as demonstrated by the exposure of cells to P38 specific inhibitor SB203580. Then, we intended to observe the effect of both Lep and p38 specific inhibitor SB203580 over time at 24, 48 and 72h. Results showed a progressive increase of NO levels at 48 and 72 hours after cytokine treatment (Fig. 4c). Pre-treatment of cells with Lep 500nM

reduced significantly the NO production, markedly at 72h, and the presence of SB203580 resulted in the significant inhibition in NO release at the same time, as measured by Griess method (Figure 4c). Overall, these results demonstrate the involvement of P38 MAPK in NO production induced by cytokines, and a specific inhibitory effect of Lep on this pathway.

Moreover, the present results show a significant activation of STAT-3 in glial cells exposed to cytokines (Figure 5). Downstream the Lep receptor STAT-3 can be also modulated by Lep signalling, although in the present results we only observed a significant decrease in phospho-STAT-3 in cells exposed to Lep. Results depicted in Figure 5 show that pre-treatment of glial cells with Lep (500nM) prevents STAT-3 activation, by phosphorylation at Tyr 705, induced by the exposure to cytokines. Since STAT-3 also has anti-apoptotic effects, it seems controversial that its inactivation by Lep could protect cells from apoptosis.

Leptin decreased the apoptosis induced by pro-inflammatory cytokines in glial cells

Results from our experiments shown the effects of Lep on mitochondrial MnSOD2 content as well as in cytochrome-c (Cyt-c) release (Figure 6). Pre-treatment of glial cells with 500nM Lep, before incubation with pro-inflammatory cytokines, significantly reduced both MnSOD2 and Cyt-c content, in agreement with the observed reduction in apoptosis. Furthermore, our results shown that despite the apparently controversial effects of Lep on apoptosis in relation with STAT-3 activity, this adipokine is in fact able to reduce the activity of the effector caspase-3, as shown by western-blot detection of a reduction in the content of the cleaved, and active, form of caspase-3 (Figure 7).

Finally, Lep is able to decrease the content of the pro-apoptotic Bax (Figure 8b), whereas increases the content of the anti-apoptotic molecule Bcl-2 and, then, contributing to the viability of glial cells (Figure 8a and 8b).

Discussion

After 20 years of research on Lep, the first reference describing the neuroprotective activity of this adipokine come from the year 2001, on a primary neuronal culture model exposed to NMDA-induced cell death [28]. Later on, Erbayat-Altay and colleagues describe that Lep was able to prevent hippocampal neuronal damage, in an experimental model of epilepsy [30]. Despite these initial papers, today persist the controversial about roles of Lep in inflammatory processes in brain.

There is an increasing evidence that leptin is involved in the pathogenesis of inflammatory and autoimmune processes. Leptin may be considered as a therapeutic target in some clinical situations [22]. By contrast, previous studies have reported that Lep stimulates the secretion of anti- and pro-inflammatory cytokines and it seems widely demonstrated that not only Lep, but also adipokines and circulating food-intake controlling metabolites, are capable of reducing glial activation in models of excitoxicity, despite the molecular mechanisms are not well understood [31, 32].

Results from the present research show a significant anti-inflammatory activity of Lep against treatments performed with cytokines TNFα, IL1β and IFNγ, in mixed glial cell cultures from C57BL/6 mice pups. We describe a significant reduction in NO production, from measured nitrite released into the culture medium, using the Griess reagent, and related to Lep exposure. Once again, some reports evidence a controversial relationship between Lep and oxidative stress, but suggest a possible interaction of reactive oxygen species and Lep in the regulation of energy metabolism in obesity. There is growing evidence that low-grade chronic inflammatory state, associated with obesity, could play a key role in disrupting Lep signalling [33,34].

Our data suggest that immunomodulatory effect of Lep on glial cell activation through iNOS inhibition might partly explain its central role in brain neuroprotective action. NO produced by iNOS seems to be a key mediator of the glial activation that induces neuronal death [7,18, 36, 37]. High levels of NO exert their toxic effects through multiple mechanisms, including lipid peroxidation, mitochondrial damage, protein nitration and oxidation, depletion of antioxidant reserves, activation or inhibition of various signalling pathways, and DNA damage [1,11 12, 14].

Thus, in the present study we demonstrated that Lep counteracted both the overexpression of iNOS and the production of NO induced by pro-inflammatory cytokines.

The observed inhibition is proportional to Lep concentration and can be related to the effect of Lep on P38 MAPK pathway. It is well known that p38 MAPK is activated by a variety of cellular stresses including inflammatory cytokines, lipopolysaccharide, UV light and growth factors. In fact, in the present study the exposure of glial cells to both cytokines and SB203580 resulted in a significant reduction in NO production and, then, we can conclude that Lep was able to inhibit P38 MAPK specifically, as can be shown by the exposure of cells to a P38 specific inhibitor SB203580.

This observation can be relevant since experiments with neurotoxic molecules, such as 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine and β-amyloid, have been reported that increase the expression if iNOS in glial cells [11, 12, 38]. Indeed, in amyotrophic lateral sclerosis, iNOS immunoreactivity is showed in activated astrocytes [39]. Then, the inhibitory effects of Lep on iNOS activity could be important to prevent oxidative damage in mitochondria and the concomitant activation of apoptosis, giving support to the anti-oxidant potential of this compound and to its potential application in the treatment of neurological disorders.

Activation of STAT-3 is evident glial cells, mainly astrocytes, under certain neuropathological conditions by phosphorylation at Tyr 705, which induces dimerization

and nuclear translocation, where favors the transcription of target genes [40-43]. Our data demonstrated that cytokine treatment induces a significant effect on STAT-3 phosphorylation in glial cells. The inhibitory effects of Lep on p-STAT-3 could contribute to reduce the inflammatory and inhibition of the apoptotic processes [41-44].

Furthermore, it also has been described that Lep could significantly decrease the apoptosis of astrocytes with ischemia/hypoxia injury, related to the increase of bcl-2 expression and the decrease of bax caspase-3 expression levels [44]. The present research shows that Lep prevents mitochondrial Cyt-c release and reduces MnSOD2 content. These data are in agreement with previous findings that have been described on an *in vitro* Type II Alveolar Epithelial Cells model [45]. In fact, several preclinical studies suggest that Lep has neuroprotective actions by inhibiting apoptotic cell death, by preventing glutamatergic cytotoxicity and protecting against oxidative stress [24]. In agreement with these previous data, our results shown that Lep is able to reduce the activity of the effector caspase-3. Leptin also seems to act on the equilibrium Bax/Bcl-2 activity. Whereas Lep is able to decrease the content of the proapoptotic Bax, increases the content of the anti-apoptotic molecule Bcl-2, although in a slightly significant manner.

Overall, our data suggest that Lep acts as an anti-inflammatory and antiapoptotic factor in glial cells exposed to pro-inflammatory cytokines, exerting its function at different levels: acting on p38 MAPK pathway and reducing its activity and NO production; acting on mitochondrial drivers of apoptosis and reducing their content, like Cyt-c; and reducing the activity of the executioner caspase-3. Then, for all these reasons, we propose that Lep may be a promising therapeutic strategy in neuroinflammatory associated brain disorders.

DISCLOSURE STATEMENT

The authors declare no competing financial interests.

ABBREVIATIONS

AD: Alzheimer's disease

AMPK: AMP-activated protein kinase

ATF: ATP protein fusion

BBB: Brain blood barrier

CNS: Central nervous system

CYT: Cytokines

Cyt-c: Cytochrome c

IFNγ: Interferon-gamma

IL1β: Interleukin 1-beta

iNOS: Nitric oxide synthase

Lep: Leptin

LepR (or ObRb): Leptin receptors

MAPK: MAP Kinase

MPTP: 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine

NO: Nitric oxide

ObRb: Leptin receptor

PPARs: Peroxisome proliferator-activated receptors

STAT3: Signal transducers and activators of transcription

SOCS3: Suppressor of cytokine signalling 3

TNFα: Tumor necrosis factor-alpha

Authors declare no conflict of interests

This study was funded by grant 2009/SGR00853 from the Generalitat de Catalunya (autonomous government of Catalonia), by grants SAF2011-23631 from the Spanish Ministerio de Ciencia e Innovación.

REFERENCES

[1] Khandelwal PJ, Herman AM, Moussa CE. Inflammation in the early stages of neurodegenerative pathology. J Neuroimmunol 2011;238:1-11.

[2] Smith JA, Das A, Ray SK, Banik NL. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. Brain Res Bull 2012;87:10-20.

[3] Azizi G, Mirshafiey A. The potential role of proinflammatory and antiinflammatory cytokines in Alzheimer disease pathogenesis. Immunopharmacol Immunotoxicol 2012;34: 881-895.

[4] Farfara D, Lifshitz V, Frenkel D. Neuroprotective and neurotoxic properties of glial cells in the pathogenesis of Alzheimer's disease. J Cell Mol Med 2008;12:762–780.

[5] Rubio-Perez JM, Morillas-Ruiz JM. A review: Inflammatory process in alzheimer's disease, role of cytokines. Scientific World Journal 2012;75:63-57.

[6] Suzumura A, Takeuchi H, Zhang G, Kuno R, Mizuno T. Roles of glia-derived cytokines on neuronal degeneration and regeneration. Ann N Y Acad Sci 2006;1088:219-229.

[7] Bhat NR, Feinstein DL, Shen Q, Bhat AN. P38 mapk-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells. Roles of nuclear factors, nuclear factor kappa b, camp response element-binding protein, ccaat/enhancer-binding protein-beta, and activating transcription factor-2. J Biol Chem 2002; 277:29584-29592.

[8] Bhat NR, Zhang P, Bhat AN. Cytokine induction of inducible nitric oxide synthase in an oligodendrocyte cell line: Role of p38 mitogen-activated protein kinase activation. J Neurochem 1999;72: 472-478.

[9] Verkhratsky A. Physiology of neuronal-glial networking. Neurochem Int 2010;57:332-343.

[10] Verkhratsky A, Kirchhoff F. Glutamate-mediated neuronal-glial transmission. J Anat 2007; 210:651-660.

[11] Brown GC. Mechanisms of inflammatory neurodegeneration: Inos and nadph oxidase. Biochem Soc Trans 2007;35:1119-1121.

[12] Brown GC. Nitric oxide and neuronal death. Nitric Oxide 2010;23:153-165.

[13] Chen M, Sun HY, Li SJ, Das M, Kong JM, Gao TM. Nitric oxide as an upstream signal of p38 mediates hypoxia/reoxygenation-induced neuronal death. Neurosignals 2009;17:162-168.

[14] Saha RN, Pahan K. Signals for the induction of nitric oxide synthase in astrocytes. Neurochem Int 2006;49:154-163.

[15] Saha RN, Pahan K. Regulation of inducible nitric oxide synthase gene in glial cells. Antioxid Redox Signal 2006;8:929-947.

[16] Brown GC, Neher JJ. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. Mol Neurobiol 2010;41:242-247.

[17] Kaminska B, Gozdz A, Zawadzka M, Ellert-Miklaszewska A, Lipko M. Mapk signal transduction underlying brain inflammation and gliosis as therapeutic target. Anat Rec (Hoboken) 2009;292:1902-1913.

[18] Sastre M, Klockgether T, Heneka MT. Contribution of inflammatory processes to alzheimer's disease: Molecular mechanisms. Int J Dev Neurosci 2006;24:167-176.

[19] Sheng W, Zong Y, Mohammad A, Ajit D, Cui J, Han D, Hamilton JL et al. Pro-inflammatory cytokines and lipopolysaccharide induce changes in cell morphology, and upregulation of erk1/2, inos and spla(2)-iia expression in astrocytes and microglia. J Neuroinflammation 2011;8:121.

[20] Martin SS, Qasim A, Reilly MP. Leptin resistance: a possible interface of inflammation and metabolism in obesity-related cardiovascular disease. J Am Coll Cardiol 2008;52:1201–1210.

[21] Borges B, Rorato RC, Uchoa ET, Marangon PB, Elias CF, Antunes-Rodrigues J et al. Protein tyrosine phosphatase-1B contributes to LPS-induced leptin resistance in male rats. Am J Physiol Endocrinol Metab 2015;308:40-50.

[22] Fernandez-Riejos P, Najib R, Santos-Alvarez J, Martín-Romero C, Pérez-Pérez A, González-Yanes C et al. Role of Leptin in the Activation of Immune Cells. Mediators of Inflammation 2010.

[23] Otero M, Lago R, Gomez R, Dieguez C, Lago F, Gomez-Reino J et al. Towards a pro-inflammatory and immunomodulatory emerging role of leptin. Rheumatology 2006;45:944–950.

[24] Arnoldussen I, Kiliaan A, Gustafson D. Adipokines interact with the brain. Eur Neuropsychopharmacol 2014;24:1982–1999.

[25] Greco SJ, Hamzelou A, Johnston JM, Smith MA, Ashford JW, Tezapsidis N. Leptin boosts cellular metabolism by activating AMPK and the sirtuins to reduce tau phosphorylation and β-amyloid in neurons. Biochem Biophys Res Commun 2011;14:170-174.

[26] McGregor G, Malekizadeh Y, Harvey J. Minireview: food for thought: regulation of synaptic function by metabolic hormones. Mol Endocrinol 2015; 29:3-13.

[27] Deumens R, Koopmans GC, Den Bakker CG, Maquet V, Blacher S, Honig WM et al., Alignment of glial cells stimulates directional neurite growth of CNS neurons in vitro. Neuroscience 2004;125:591-604.

[28] Vilar A, de Lemos L, Patraca I, Martínez N, Folch J, Junyent F et al. Melatonin suppresses nitric oxide production in glial cultures by pro-inflammatory cytokines through p38 MAPK inhibition. Free Radic Res 2014;48:119-28.

[29] Dicou E, Attoub S, Gressens P. Neuroprotective effects of leptin in vivo and in vitro. Neuroreport 2001;12:3947-51.

[30] Erbayat-Altay E, Fessler AJ, Gallagher M, Attarian HP, Dehdashti, Vahle VJ et al. Correlation of severity of FDG-PET hypometabolism and interictal regional delta slowing in temporal lobe epilepsy. Epilepsia 2005;46:573-576.

[31] Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML,Wang DJ, et al. Leptin regulates proinflammatory immune responses. FASEB J 1998;12:57–65.

[32] Beltowski J, Wojcicka G, Marciniak A, Jamroz A. Oxidative stress, nitric oxide production, and renal sodium handling in leptin-induced hypertension. Life Sci 2004;74: 2987–3000.

[33] Sáinz N, Barrenetxe J, Moreno-Aliaga MJ, Martínez JA. Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. Metabolism 2015;64:35-46.

[34] Sáinz N, González-Navarro CJ, Martínez JA, Moreno-Aliaga MJ. Leptin signaling as a therapeutic target of obesity. Expert opin Ther Targets 2015;19:893-909.

[35] Ji RR, Suter MR. P38 mapk, microglial signaling, and neuropathic pain. Mol Pain 2007;3:33.

[36] Graeber MB, Li W, Rodriguez ML. Role of microglia in cns inflammation. FEBS Lett 2011; 585:3798-3805.

[37] Ionov M, Burchell V, Klajnert B, Bryszewska M, Abramov AY. Mechanism of neuroprotection of melatonin against beta-amyloid neurotoxicity. Neuroscience 2011;180:229-237.

[38] Feng Z, Zhang JT. Protective effect of melatonin on beta-amyloid-induced apoptosis in rat astroglioma c6 cells and its mechanism. Free Radic Biol Med 2004;37:1790-1801.

[39] Barbeito LH, Pehar M, Cassina P, Vargas MR, Peluffo H, Viera L et al. A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis. Brain Res Brain Res Rev 2004;47:263-274.

[40] Pérez-Capote K, Serratosa J, Solà C. Glial activation modulates glutamate neurotoxicity in cerebellar granule cell cultures. Glia 2004;45:258-68.

[41] Kurrimbux D, Gaffen Z, Farrell CL, Martin D, Thomas SA. The involvement of the blood-brain and the blood-cerebrospinal fluid barriers in the distribution of leptin into and out of the rat brain. Neuroscience 2004;123:527-36.

[42] Folch J, Patraca I, Martínez N, Pedrós I, Petrov D, Ettcheto M, Abad S, Marin M, Beas-Zarate C, Camins A. The role of leptin in the sporadic form of Alzheimer's disease. Interactions with the adipokines amylin, ghrelin and the pituitary hormone prolactin. Life Sci. 2015; 140:19-28.

[43] Wang YN, Yang M, Yu LH, Guo J, Chen N, He L. Leptin play the key role in astroglial differentiation of mouse neural stem cells and regulated theSTAT3 signaling through Jak-STAT3 pathway. Sichuan Da Xue Bao Yi Xue Ban 2014;45:552-556.

[44] Si YL, Zhang JY, Hao XH, Deng ZH, Xue H, Yan GT. Effects of leptin on apoptosis of rat cerebral astrocytes with ischemia/hypoxia injury. Sichuan Da Xue Bao Yi Xue Ban 2010;41:403-407.

[45] Chen H, Liang ZW, Wang ZH, Zhang JP, Hu B, Xing XB et al. Akt Activation and Inhibition of Cytochrome-c Release: Mechanistic Insights Into Leptin-Promoted Survival of Type II Alveolar Epithelial Cells. J Cell Biochem 2015;116:2313-24.

FIGURE LEGENDS

FIGURE LEGENDS

Figure 1. (**A**) Western-blot determination of Ob-R expression in cell culture at 9^{th}, 15^{th} and 21^{th} day. The intensity of the bands was determined by densitometric analysis, and the data were normalized using β-actin as an internal control. Results shown a significant decrease in the receptor expression up to 9^{th} day after plating in 60 mm dishes (***p<0.001 when comparing expression at 15^{th} and 21^{th} days with the observed at 9^{th} day). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test. Data were normalized using β-actin as internal controls. (**B**) The presence of Lep receptors was determined by immunohistochemistry with anti Ob-R (H-300) sc-8325 antibody (magnification: X200).

Figure 2. Cytokine effects on cell viability. (**A**) Treatment of mixed glial cell cultures with three cytokines TNFα, IL1β and IFNγ (CYT) for 48h, significantly reduced cell viability as shown by MTT determination. The increased formazan formation induced by CYT was partially reversed by Lep (500nM). Control bar was taken as 100%. Each point is the mean ± SEM of three or four independent experiments (^{+}p<0.05 when comparing CYT group vs control; *p<0.05 when comparing Lep alone and Lep+CYT groups with respect to CYT group). (**B**)The graph illustrates results from Hoechst 33342 staining of nuclei. Images show increased apoptosis in glial cells with chromatin condensation in the nuclei. Measures were performed with a Reader: Ascent Labsystems Flouroskan Ex: 350 nm Em: 463 nm. (^{+++}p<0.001 when comparing CYT group vs control; ***p<0.001 when comparing Lep alone and Lep+CYT groups with respect to CYT group). Images (**C** to **E**) show the presence of apoptotic, picnotic, nuclei in cultures exposed to cytokines at 48h (magnification: X200). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 3. (**A**) Figure shows a significant increase in nitrite levels (μM) measured by Griess method, in glial cells after 48 hours of treatment with pro-oxidant cytokines. The pre-incubation of cultures with Lep for 1h before the exposure to cytokines partially reversed the nitrite production. The exposure to Lep concentrations of 50, 100 and 500 nM demonstrated a protective anti-inflammatory dose-dependent effect. The Control bar was taken as 100%. Each point is the mean ± SEM of three or four independent experiments (^{+++}p<0.001 when comparing CYT group vs control; *p<0.05 and ***p<0.001 when comparing Lep+CYT groups [Lep ranging from 50 to 500nM] with respect to CYT group). (**B**) The content of iNOS in glial cells 48h after cytokine treatment was significantly increased, and also pre-treatment with 500nM Lep reversed this effect. An image from Western-blot, normalized with β-actin, is shown below (^{+++}p<0.001 CYT group compared with control; **p<0.01 when comparing Lep alone and Lep+CYT groups with respect to CYT group). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 4. Western-blots against (**A**) phospho-P38 (Thr180/Tyr182) form and (**B**) total P38 after treatment with CYT and Lep. Results show that pre-incubation with Lep

causes a significant reduction in the content of the active phospho-P38 form. All Western-blots were normalized with β-tubulin. Control bar was taken as 100%. Each point is the mean ± SEM the mean of three independent experiments (^{+}p<0.05 and ^{+++}p<0.001 when comparing CYT group vs control; **p<0.01 when comparing Lep alone and Lep+CYT groups with respect to CYT group). (**C**) Time-course of NO release at 24, 48 and 72 hours, after treatment with cytokines, and measured by Griess method. Graph shows a significant effect of both P38 MAPK inhibitor SB203580 and Lep (both 500nM) in reducing this effect up to 48h (**p<0.01 when comparing CYT vs either Lep+CYT or Inhibitor+CYT groups). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 5. Results from Western-blots against (**A**) STAT-3 and (**B**) phospho-STAT-3 (Tyr 705) 48 hour after addition of CYT. Pre-treatment with Lep reduced significantly the content of phospho-STAT-3 induced by the exposure to CYT. Lep itself showed a great variability in its capacity to activate the phosphorylation of STAT-3. The data from three independent experiments are expressed as the mean ± SEM. Data were normalized using β-actin as internal controls. (^{+++}p<0.001 when comparing CYT group vs control; **p<0.01 when comparing Lep alone and Lep+CYT groups with respect to CYT group). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 6. Effects of Lep in apoptosis. Results from Western-blots against Cyt-c (**A**) and MnSOD2 (**B**) indicated significant changes in mitochondrial key enzymes involved in activation of apoptosis. Pre-treatment with Lep (500nM) 60 minutes before treatment with CYT, reduced significantly this pro-apoptotic effect. Data were normalized using β-actin as internal control. Control was taken as 100 %. Each point is the mean ± SEM the mean of three independent experiments (^{+++}p<0.001 when comparing CYT group vs control; **p<0.01 when comparing Lep alone and Lep+CYT groups with respect to CYT group). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 7. Effects of Lep pre-treatment on caspase-3 activation (**A**). Astrocytes were incubated with leptin 500nM in the absence or presence of cytokines for 48h. The activation of the cleaved and activated form of caspase-3 was determined by Western blotting (**B**). The density of the band of cleaved caspase-3 under the indicated treatments was quantified and divided by the value of the density of GAPDH. The percentages of cleaved caspase-3 levels in treated cells are normalized vs control. Control was taken as 100 %. Each point is the mean ± SEM the mean of three independent experiments (^{+}p<0.05 and ^{+++}p<0.001 when comparing CYT group vs control; *p<0.05 and ***p<0.001 Lep alone and Lep+CYT groups with respect to CYT). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 8. An inverse relationship in the expression of Bcl-2/Bax was found. (**A**) Bcl-2 has been considered to be a pro-survival mitochondrial molecule, and resulted stimulated by Lep (500nM), indicating a protection against inflammation and apoptosis. Control was taken as 100 %. Each point is the mean ± SEM the mean of three independent experiments (*p<0.05 Lep alone and Lep+CYT groups with respect to CYT). (**B**) By contrast, the activation of the pro-apoptotic Bax was reversed by the

exposure to Lep 500nM. Each point is the mean ± SEM the mean of three independent experiments ($^{+++}p<0.001$ when comparing CYT group vs control; ***$p<0.001$ when comparing Lep alone and Lep+CYT groups with respect to CYT group). (**C**) The relative protein expression levels of Bcl-2 and Bax were measured (**$p<0.01$ when comparing Lep alone and Lep+CYT groups with respect to CYT group). Statistical differences were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 1

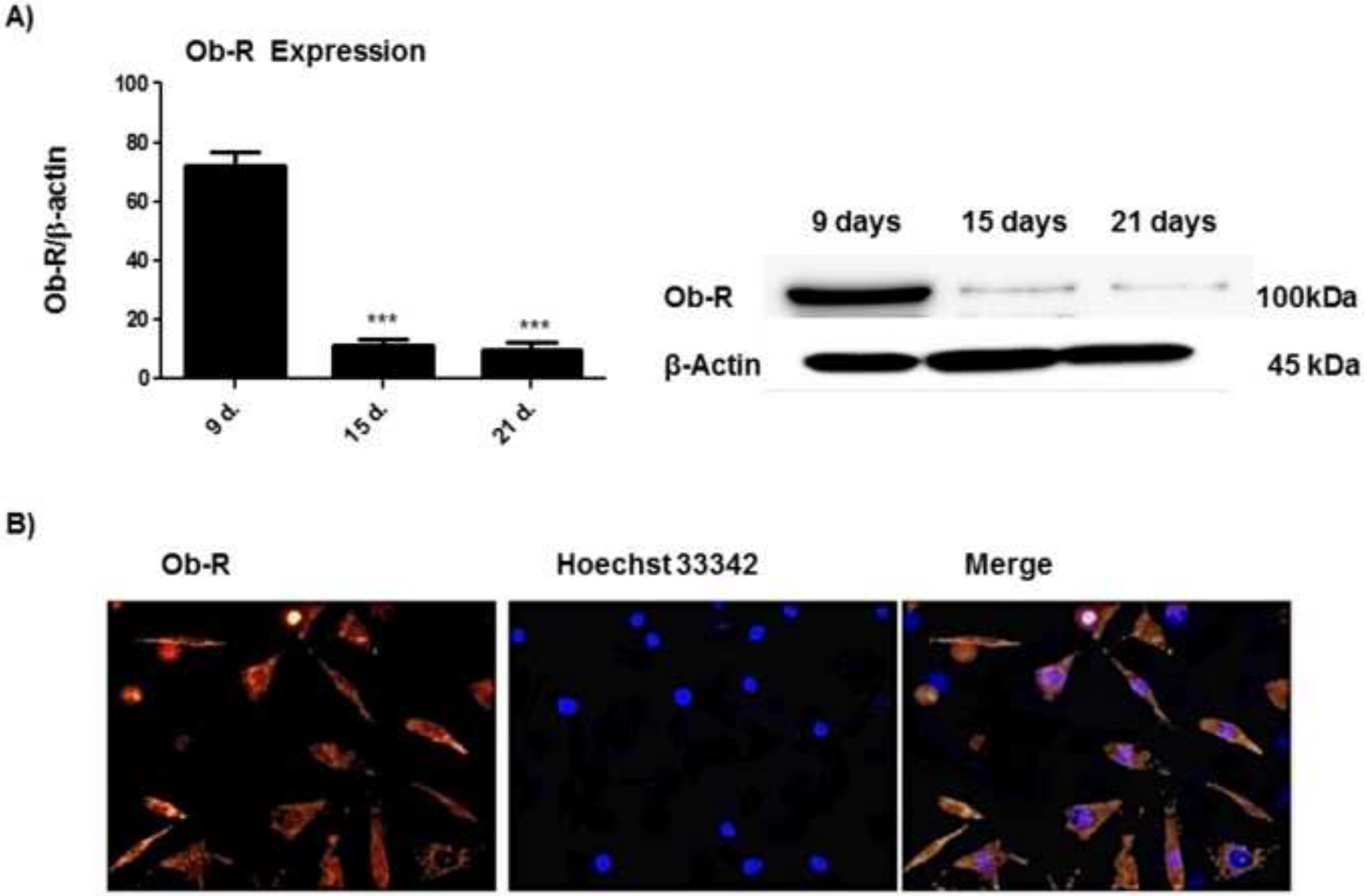

A)
Ob-R Expression
Ob-R/β-actin
100
80
60
40
20
0
***
***
9 d.
15 d.
21 d.
9 days
15 days
21 days
Ob-R
100kDa
β-Actin
45 kDa
B)
Ob-R
Hoechst 33342
Merge

Figure 2

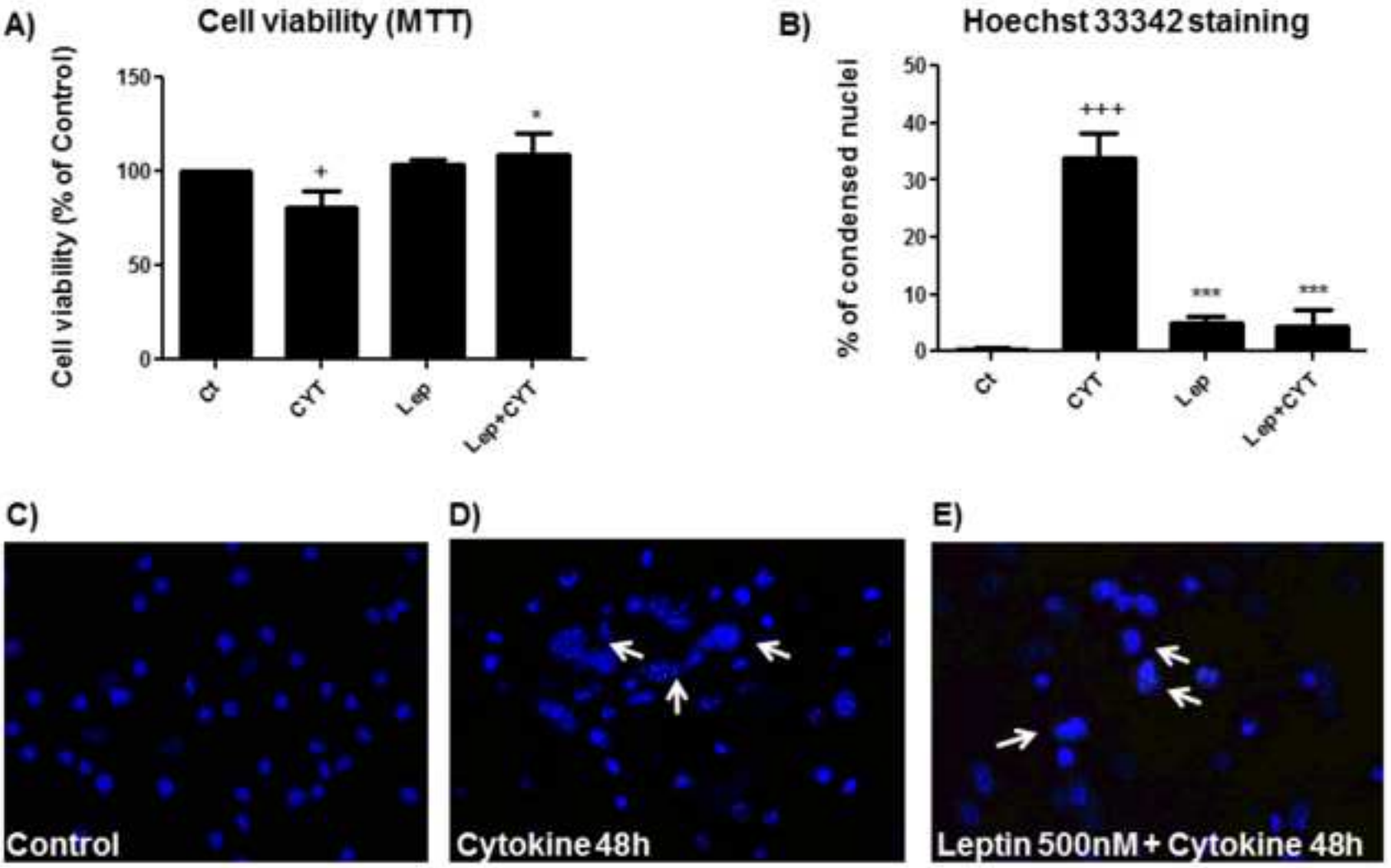

A)
Cell viability (MTT)
Cell viability (% of Control)
150
100
50
0
+
*
Ct
CYT
Lep
Lep+CYT
B)
Hoechst 33342 staining
% of condensed nuclei
50
40
30
20
10
0
+++
***
***
Ct
CYT
Lep
Lep+CYT
C)
Control
D)
Cytokine 48h
E)
Leptin 500nM + Cytokine 48h

Figure 3

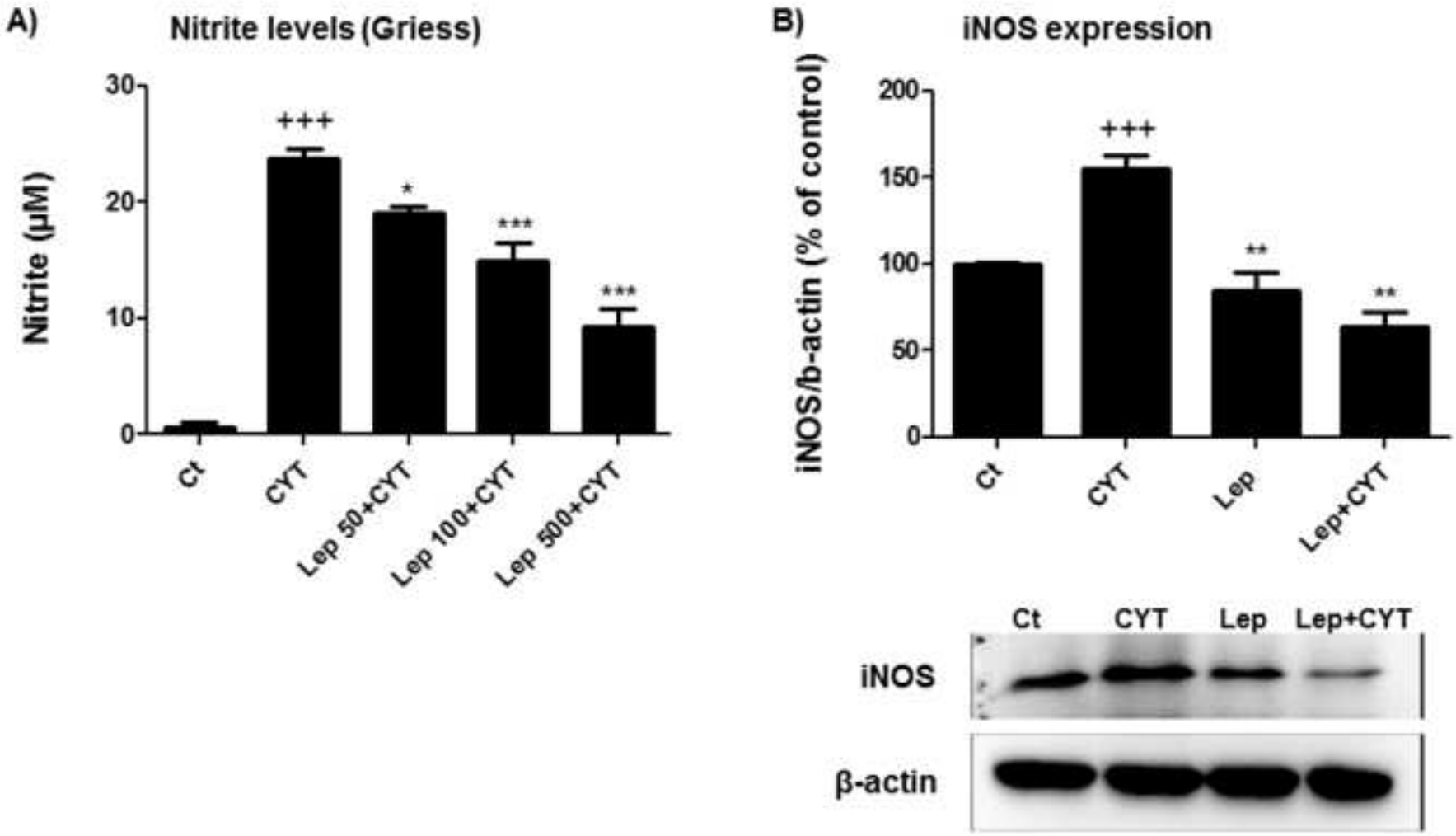

A)
Nitrite levels (Griess)
Nitrite (μM)
30
20
10
0
+++
*
***
***
Ct
CYT
Lep 50+CYT
Lep 100+CYT
Lep 500+CYT
B)
iNOS expression
iNOS/b-actin (% of control)
200
150
100
50
0
+++
**
**
Ct
CYT
Lep
Lep+CYT
Ct
CYT
Lep
Lep+CYT
iNOS
β-actin

Figure 4

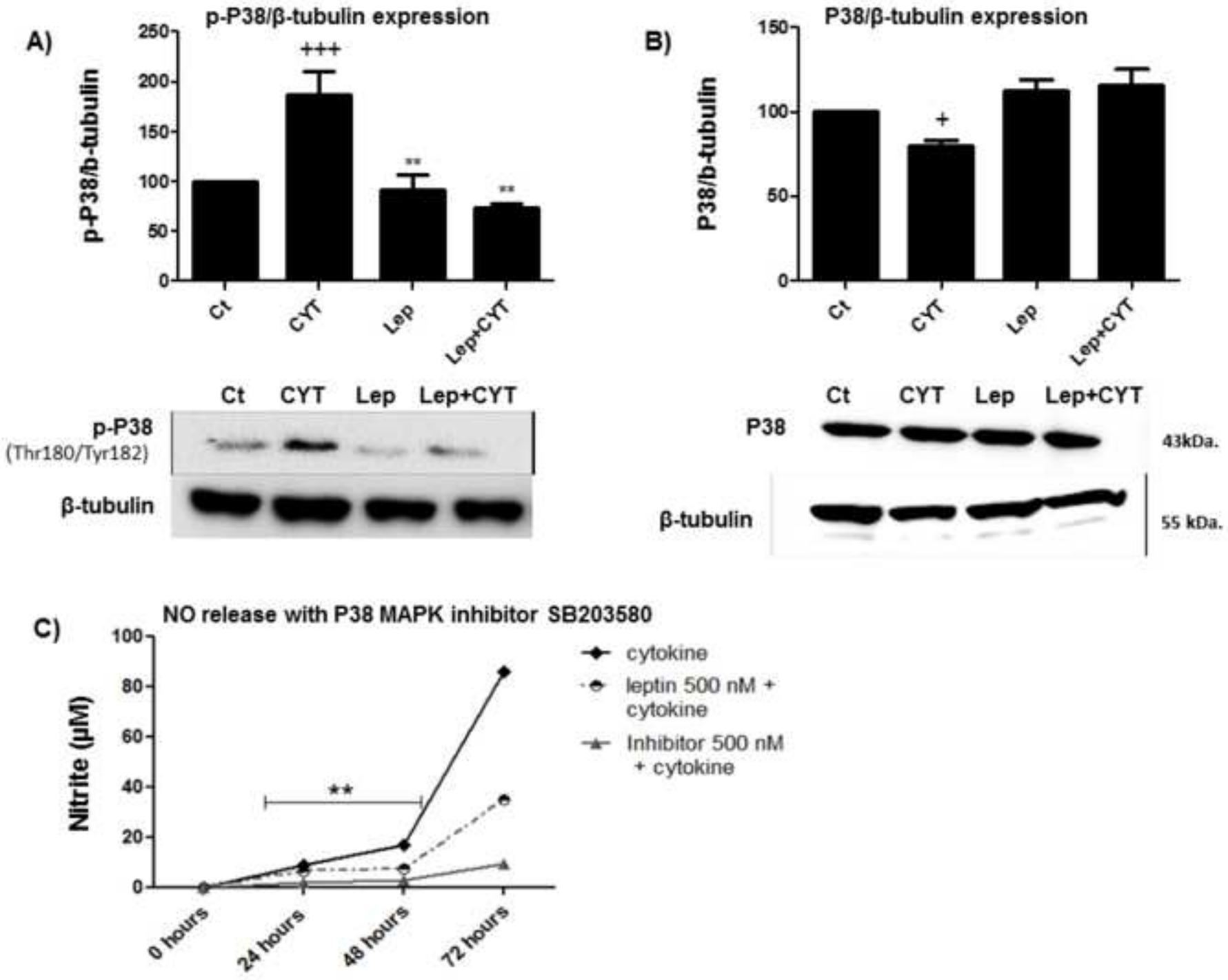

A)
p-P38/β-tubulin expression
p-P38/b-tubulin
250
200
150
100
50
0
+++
**
**
Ct
CYT
Lep
Lep+CYT
Ct CYT Lep Lep+CYT
p-P38
(Thr180/Tyr182)
β-tubulin
B)
P38/β-tubulin expression
P38/b-tubulin
150
100
50
0
+
Ct
CYT
Lep
Lep+CYT
Ct CYT Lep Lep+CYT
P38
43kDa.
β-tubulin
55 kDa.
C)
NO release with P38 MAPK inhibitor SB203580
Nitrite (μM)
100
80
60
40
20
0
**
0 hours
24 hours
48 hours
72 hours
cytokine
leptin 500 nM + cytokine
Inhibitor 500 nM + cytokine

Figure 5

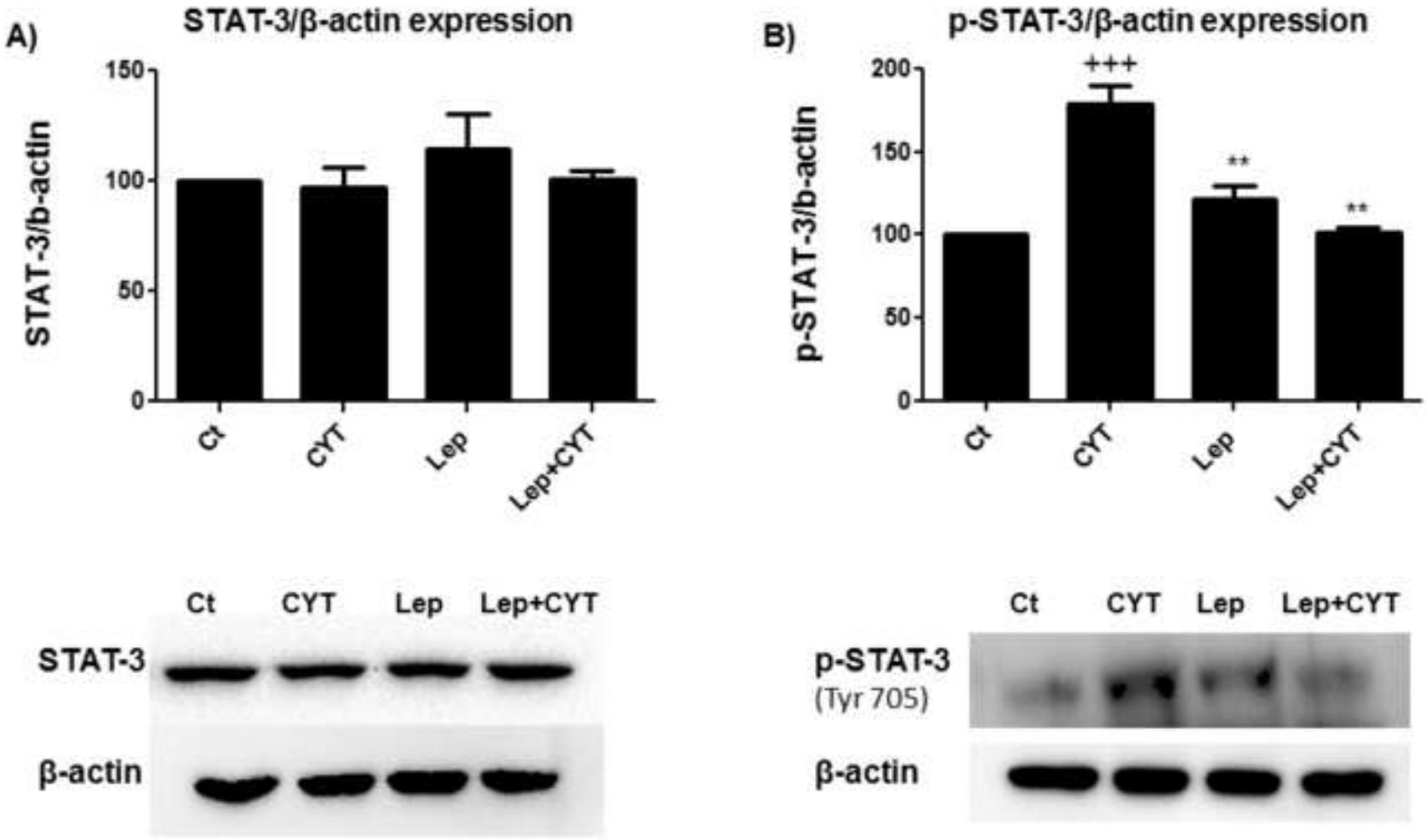

A)
STAT-3/β-actin expression
STAT-3/b-actin
150
100
50
0
Ct
CYT
Lep
Lep+CYT
B)
p-STAT-3/β-actin expression
p-STAT-3/b-actin
200
150
100
50
0
+++
**
**
Ct
CYT
Lep
Lep+CYT
Ct CYT Lep Lep+CYT
STAT-3
β-actin
Ct CYT Lep Lep+CYT
p-STAT-3
(Tyr 705)
β-actin

Figure 6

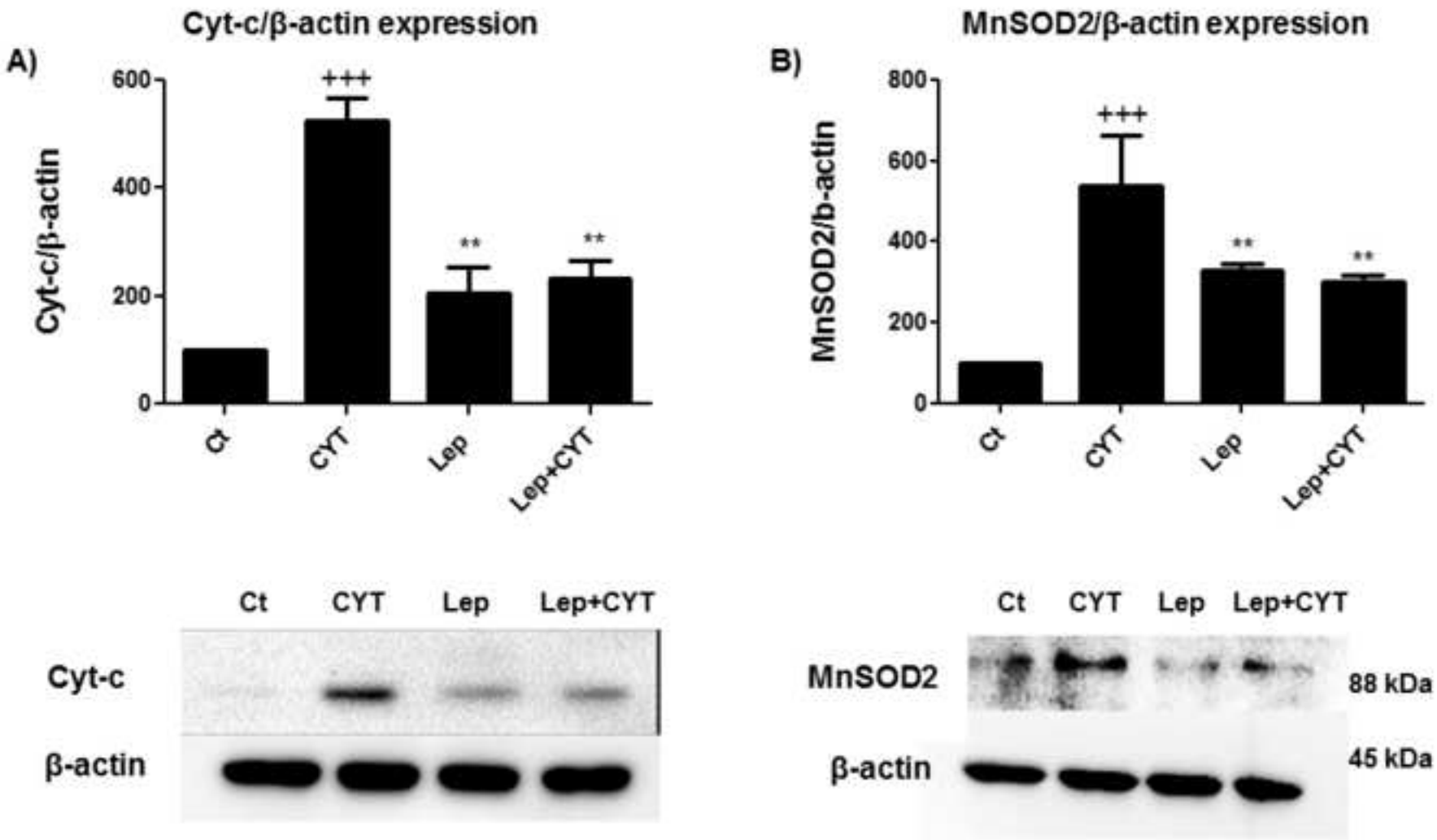

A)
Cyt-c/β-actin expression
Cyt-c/β-actin
600
400
200
0
+++
**
**
Ct
CYT
Lep
Lep+CYT
Ct
CYT
Lep
Lep+CYT
Cyt-c
β-actin
B)
MnSOD2/β-actin expression
MnSOD2/b-actin
800
600
400
200
0
+++
**
**
Ct
CYT
Lep
Lep+CYT
Ct
CYT
Lep
Lep+CYT
MnSOD2
88 kDa
β-actin
45 kDa

Figure 7

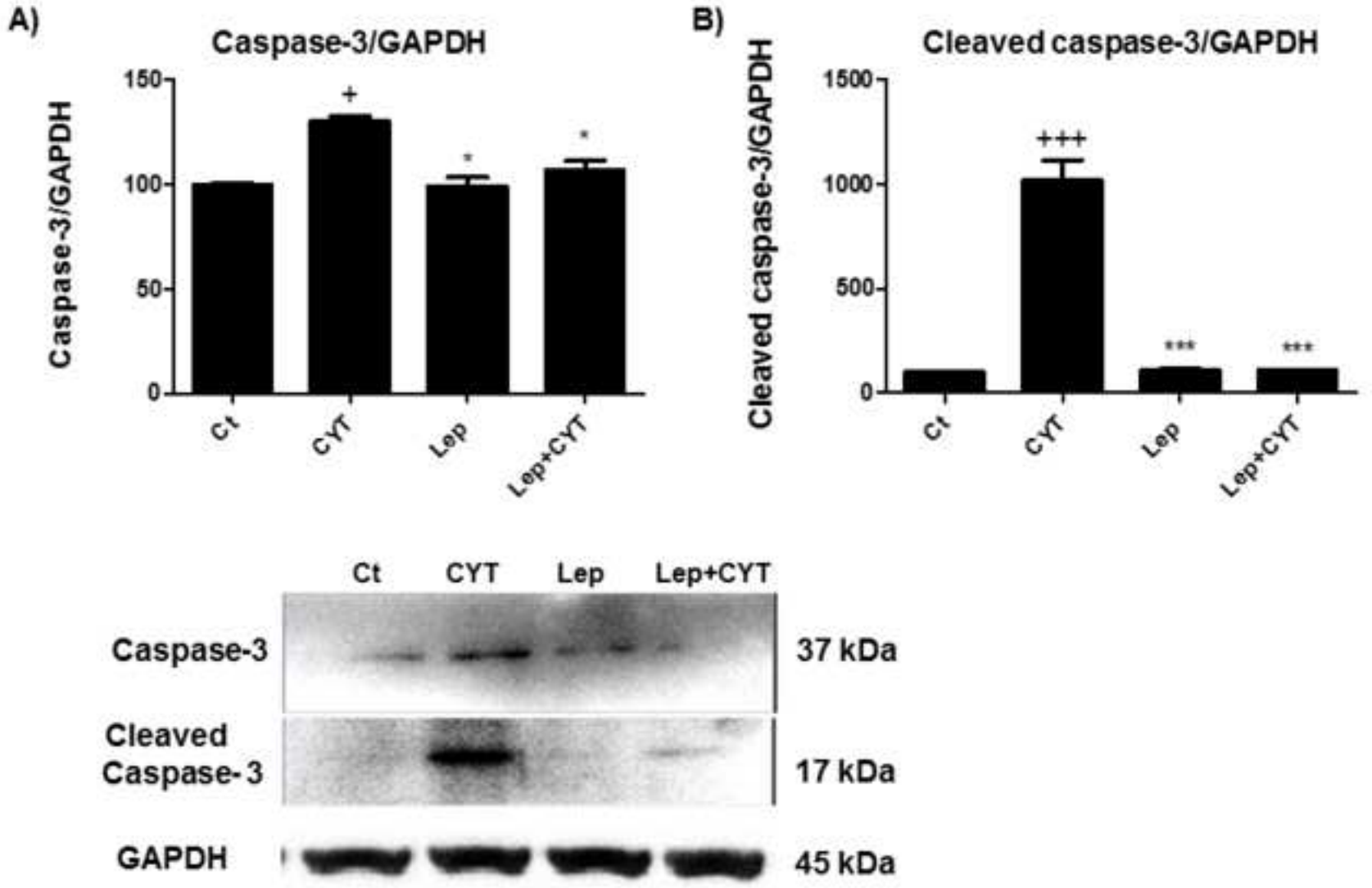
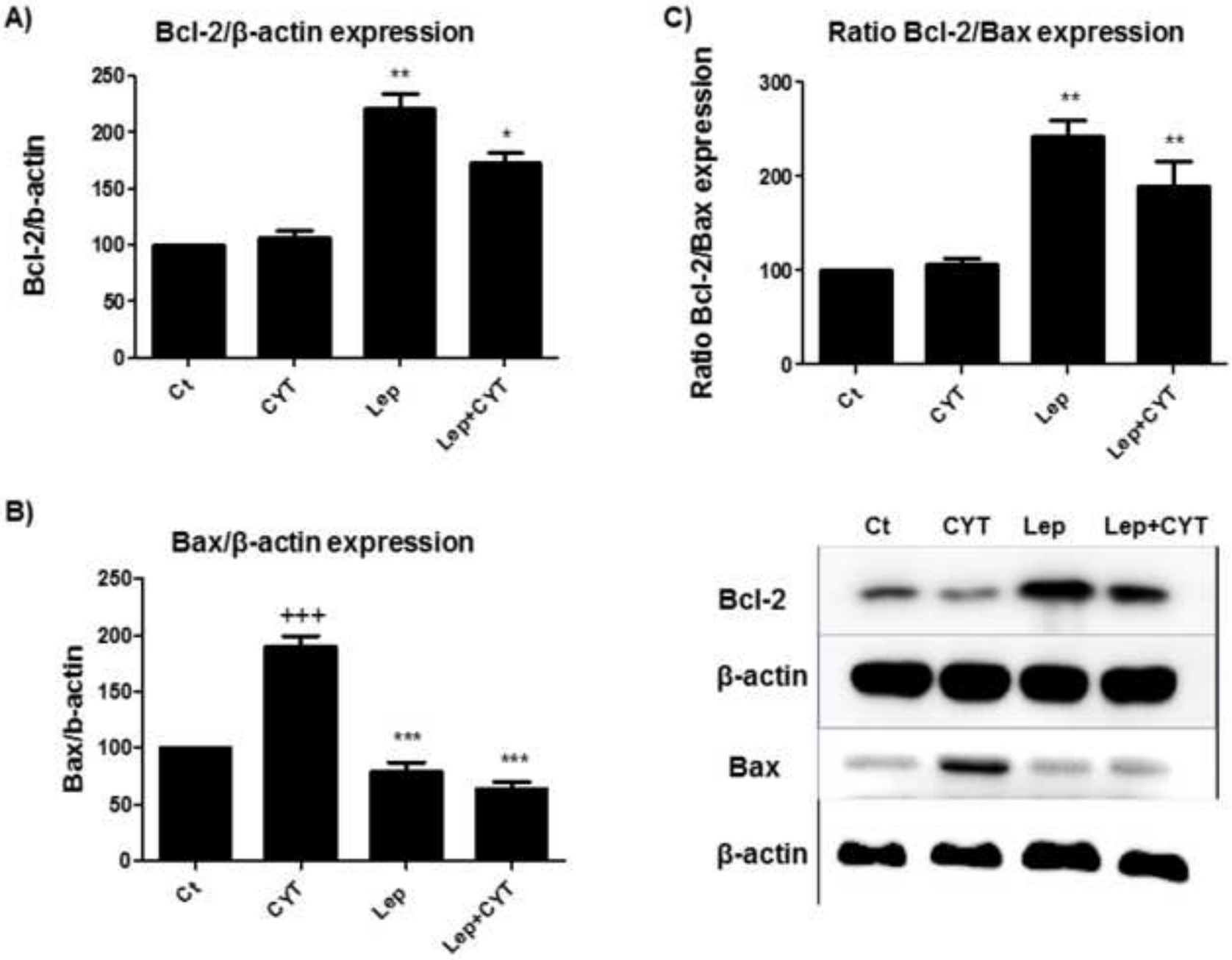

A)
Caspase-3/GAPDH
Caspase-3/GAPDH
150
100
50
0
+
*
*
Ct
CYT
Lep
Lep+CYT
B)
Cleaved caspase-3/GAPDH
Cleaved caspase-3/GAPDH
1500
1000
500
0
+++
***
***
Ct
CYT
Lep
Lep+CYT
Ct
CYT
Lep
Lep+CYT
Caspase-3
37 kDa
Cleaved
Caspase-3
17 kDa
GAPDH
45 kDa

Figure 8


A)
Bcl-2/β-actin expression
Bcl-2/b-actin
250
200
150
100
50
0
**
*
Ct
CYT
Lep
Lep+CYT
C)
Ratio Bcl-2/Bax expression
Ratio Bcl-2/Bax expression
300
200
100
0
**
**
Ct
CYT
Lep
Lep+CYT
B)
Bax/β-actin expression
Bax/b-actin
250
200
150
100
50
0
+++
***
***
Ct
CYT
Lep
Lep+CYT
Ct
CYT
Lep
Lep+CYT
Bcl-2
β-actin
Bax
β-actin