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Time-dependent effects of systemic lipopolysaccharide injection on regulators of antioxidant defense Nrf2 and PGC-1 α in neonatal rat brain.

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Abstract

Background/Aims—Both excitotoxicity and neuroinflammation are associated with oxidative stress. One transcription factor, Nrf2 and one transcription co-factor PGC-1α, increase the endogenous antioxidant defense and can thus modulate neuronal cell death. Here we have investigated the temporal effects (24 and 72 h) of systemic (i.p.) administration of lipopolysaccharide (LPS) on the cerebral Nrf2 and PGC-1α systems.

Methods and Results—Seven-day old rat pups were injected with LPS (0.3 mg/kg). After 24 h the protein levels of γGCL-M, γGCL-C, Nrf2, PGC-1α and MnSOD were increased in parallel with decreased levels of Keap1. These effects were correlated with increased level of phosphorylated Akt and elevated acetylation of histone 4. In contrast, 72 h following LPS a decrease in the components of the Nrf2 system in parallel with an increase of Keap1 was observed. The down-regulation after 72 h correlated with phosphorylation of p38 MAPK, while there were no changes in PGC-1α and MnSOD protein levels or the acetylation/methylation pattern of histones.

Conclusion—Systemic LPS in neonatal rats induced time dependent changes in brain Nrf2 and PGC-1α that correlated well with the protective effect observed after 24 h (preconditioning) and the deleterious effects observed after 72 h (sensitizing) of systemic LPS reported earlier. Collectively, the results point towards Nrf2 and PGC-1α as a possible mechanism behind these effects.

Keywords

neuroinflammation; Nrf2; antioxidant system; PGC-1α

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Introduction

Excitotoxicity, oxidative stress and inflammation are tightly interrelated factors in the neurotoxic process in acute ischemia and chronic neurodegenerative diseases [1,2]. Down regulation of anti-oxidant systems promotes oxidative stress and inflammation caused by excitotoxicity [3]. The transcription factor nuclear factor-E2-related factor-2 (Nrf2), a member of the “cap ‘n collar” group of transcription factors, is essential for protecting cells against oxidative damage [4]. Deletion of Nrf2 makes animals over-sensitive to oxidative stress and they develop white matter damage and retinopathy spontaneously [3,5,6]. Interestingly brains from Alzheimer patients demonstrate low levels of Nrf2 in hippocampal astrocytes [7] indicating inadequate astroglial antioxidant support in this disease. Studies done in Nrf2 knockout mice show larger infarct volume after cerebral ischemia; and *t*BHQ (*tert*butylhydroquinone), an Nrf2 activator, limited the damage in the penumbra region but not in the core of infarction area in wild type animals only [8]. Similarly, the Nrf2 inducers sulphoraphane, curcumin and (-)epicatechin have shown protective effects after transient middle cerebral artery (MCA) occlusion [9-11]. The endogenous induction of the Nrf2 system in the peri-infarct region [8] is, at least partly, likely due to down-regulation of the cytoplasmatic protein Keap1 that sequesters Nrf2 [12]. Regulation of the inducible anti-oxidant system in neonatal CNS has not been studied in great detail, however, it has been shown that the Nrf2 activator sulphoraphane can protect against neonatal hypoxia-ischemia in rats [13]. Our earlier studies showed that the level of glutathione (GSH) was decreased in brain after hypoxia-ischemia [14]. In a later study we demonstrated that the reduction in GSH levels was more pronounced when the hypoxia-ischemia was preceded by inflammation induced by lipopolysaccharide (LPS) injection in postnatal day (PND) 8 rats 72 h prior to the insult [15]. Both the brain damage and the decrease in GSH were normalized by multiple injections of the anti-oxidant and GSH precursor N-acetylcysteine, indicating that GSH synthesis was reduced by the inflammation [15]. In contrast, LPS injection 24 h prior to hypoxia-ischemia *protected* against brain damage [16]. In a recent study *in vitro* we mimicked these effects of neuroinflammation *in vivo* by activating microglia with endotoxin and studied the effects of microglia conditioned medium on the Nrf2 system in astrocytes after 24 and 72 h of treatment. These studies showed that inflammation may have dual, time related, effects on the Nrf2 system that were dependent on protein kinase activation, i.e. activation of Akt, JNK and ERK stimulated the Nrf2 system whereas sustained activation of GSK3β and p38 down-regulated the system [17]. Another factor that has complementary effects on the anti-oxidants system is the peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 alpha (PGC-1α), which functions as a cofactor for many transcription factors [18]. Activation of PGC-1α results in elevated levels of Mn-superoxide dismutase (MnSOD) present in the mitochondria. Earlier it has been shown that LPS treatment increases MnSOD, both *in vitro* and *in vivo* [19,20].

Here we have employed the *in vivo* model and investigated how inflammation by systemic LPS (0.3 mg/kg of LPS (i.p.)) affects the Nrf2 and PGC-1α systems in the brain after 24 and 72 h.

Materials and Methods

Animal treatment and Tissue Preparation

Sprague-Dawley rats from our in-house colony were housed in a 12 h light-dark cycle and bred at Experimental Biomedicine (Gothenburg University, Gothenburg, Sweden) and were provided with a standard laboratory chow diet (B&K, Solna, Sweden) and drinking water *ad libitum*. All animal experiments were approved by the Ethical Committee of Gothenburg (Ethical number 264-2009) and followed the guidelines for the care and use of experimental animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Rats were injected intraperitoneally (i.p.) with saline or LPS (0.3 mg/kg) at PND 7 and brain tissue was collected at 24 h (saline group = 9, LPS group = 11) and 72 h (saline group = 9, LPS group = 11) after LPS exposure for western-blot assessment. Animals were deeply anesthetized and perfused intracardially with ice-cold saline. Brains were rapidly removed and frozen in liquid nitrogen and stored at −80°C until further use.

After thawing, ice-cold isolation buffer (50 mM Tris, pH 7.3; 2 mM EDTA, 1% protease inhibitor cocktail) was added and homogenization was performed gently with a manual homogenizer (VWR International). The cytosolic and nuclear fractions were separated according to a method described previously (Wang et al., 2003). Briefly, the homogenates were centrifuged at 800 × g at 4°C for 10 min. The pellets were washed in homogenizing buffer and recentrifuged, producing a crude nuclear pellet (P1). The supernatant was further centrifuged at 9200 × g for 15 min at 4°C, producing a crude mitochondrial fraction in the pellet (P2) and a cytosolic fraction in the supernatant (S). All fractions were stored at −80°C. P1 and S fractions were used for immunoblotting.

Western Blot Analysis

Homogenate fractions were mixed with 5X Laemmle sample buffer and boiled for 5 min. Then equal amount of protein (30 μg) were resolved on 10% SDS-PAGE in a MOPS or MES buffer (Invitrogen; Carlsbad, USA) and electroblotted at 40 V for 70 min at 4 °C to nitrocellulose (Bio-Rad; Hercules, USA). The membranes were blocked for 1 h at room temperature (RT) in 5 % (w/v) dry skimmed milk (Semper Mjölk; Sundyberg, Sweden) in TBS with 0.1 % Tween 20 (TBST). Then, the membranes were incubated overnight at 4 °C with the corresponding primary antibodies in 5% bovine serum albumin (BSA)-TBST, extensively washed with TBST solution and incubated with the correspondent secondary antibodies for 1 h at RT. Finally, the blots were rinsed and the peroxidase reaction was developed by enhanced chemiluminescence SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific; Rockford, USA). Blots were stripped in RestoreTM Plus Western Blot Stripping Buffer (Thermo Scientific; Rockford, USA) and were reprobed sequentially.

Images were captured with a Fujifilm Image Reader LAS-1000 Pro v2.6 (Stockholm, Sweden) and the different band intensities (density arbitrary units) corresponding to immunoblot detection of protein samples were quantified using the Fujifilm Multi Gauge v3.0 software (Stockholm, Sweden).

Antibodies

Anti-Histone H2B, anti-acetyl-Histone H3, anti-acetyl-Histone H4 and anti-trimethyl-Lys9-Histone H3 were from Millipore (Solna, Sweden). Anti-phospho-p38 and anti-phospho-Akt were from New England Biolabs (Beverly, USA). Anti-Nrf2 was from R&D Diagnostics (Minneapolis, USA). Anti-Keap1, anti-PGC-1α, anti-actin, anti-α-tubulin, anti-γGCL-C and anti-γGCL-M antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-MnSOD was from Lab Frontier (Seoul, South Korea). Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Vector Laboratories (Burlingame, USA).

Statistical Analysis

Results are presented as means ± standard error mean (SEM). Student's t test was used to determine statistical significance (95%; $p<0.05$). If the Levene's test for homogeneity of variances was significant then the nonparametric Mann-Whitman U test was used.

Results

Effect of 24 h systemic LPS exposure on the cerebral Nrf2 system

In our earlier studies we showed that conditioned medium from LPS activated microglia can have positive effects after 24 h but negative effects after 72 h on the Nrf2-system in astrocyte-rich cultures [21]. Similarly dual effects of LPS with protection after 24 h and sensitisation after 72 h to a hypoxic-ischemic insult was demonstrated in neonatal rats [15]. Therefore we evaluated the effects of systemic LPS after 24 and 72 h on the Nrf2 system in vivo in this study.

The effects of 24 h exposure of 7-day old rats to 0.3 mg/kg LPS (i.p.) on the components of the brain inducible Nrf2 and PGC-1α systems are illustrated in Fig 1. We first analysed the effects of LPS on cerebral Nrf2, Keap1, γGCL-C and γGCL-M protein expression (fig 1A). As shown in Fig. 1B, the densitometric analysis of western blot showed that 24 h systemic LPS induced an increased expression of Nrf2, γGCL-C and γGCL-M but reduced the level of Keap1. Similarly, 24h after LPS exposure, there was an increase in the protein levels of PGC1α and MnSOD (fig 1C) as shown in the densitometric analysis (fig 1D).

Moreover, we analysed the cytoplasmic and nuclear fractions of the brain homogenates and found that there was an increase in Nrf2 protein levels in both fractions (fig 2).

To investigate the possible mechanisms relevant to the Nrf2 related changes observed above, we further analysed changes of the MAPK signal pathway and cerebral acetylation and methylation patterns of histones. The analysis of the MAPK signalling pathways activated in the brain by the systemic administration of LPS (24 h) showed increased phosphorylation levels of Akt (fig 3A). The levels of phosphorylated p38 MAPK and JNK were below detection limits. The systemic treatment with LPS for 24 h also induced changes in the cerebral acetylation and methylation of histones. Thus, an increase in the acetylation of histone H4 and in the methylation of histone H3-K9 was detected with no changes in the acetylation pattern of H3, as revealed in figure 3C and 3D.

Effect of 72 h systemic LPS on the cerebral Nrf2 system

The effects of 72 h exposure to 0.3 mg/kg LPS (i.p.) on the components of the brain inducible Nrf2 and PGC1α systems in 7-day old rats are shown in Fig 4. We first analysed the effects of LPS on cerebral Nrf2, Keap1, γGCL-C and γGCL-M protein expression (fig. 4A). The densitometric analysis of western blot bands showed that 72 h after systemic LPS injection expression of Nrf2, γGCL-C and γGCL-M was lower, but higher levels of Keap1 were observed when compared to saline treatment (fig. 4B). Next, the protein levels of PGC1α and MnSOD were analysed (fig. 4C), however no changes were observed, as shown in the densitometric analysis (fig. 4D).

When the cytoplasmic and nuclear fractions were analysed, we found that in both fractions the protein levels of Nrf2 were reduced compared to saline control (fig. 5).

The analysis of the signalling pathways activated in the brain by the systemic administration of LPS (72 h) showed unchanged phosphorylation levels of Akt with a concomitant increase in the phosphorylation levels of p38 MAPK (fig. 6A and 6B). Both JNK and ERK1/2 were below detection limits (data not shown). Interestingly, 72h following the systemic treatment with LPS there were no changes in the cerebral acetylation and methylation pattern of histones, albeit a tendency to a lower acetylation levels of histone H3 which did not reach statistical significance (fig. 6C and 6D).

Discussion

LPS elevated the protein levels of Nrf2 and its down-stream products, γGCLM and γGCLC 24 h after systemic administration. This is in agreement with our earlier finding that systemic LPS in neonatal rats increased brain levels of Nrf2 mRNA after 24 h [22]. In addition, the complementary transcriptional co-factor PGC-1α and MnSOD, which transcription is elevated by PGC-1α, were raised at this time point. These effects point towards an induced anti-oxidant defence by LPS at 24 h after treatment. Such elevation in the anti-oxidant systems may well underlie our previously observed preconditioning effect and protection by LPS in neonatal rats at 24 h after administration [16]. This also agrees well with the induction of the Nrf2 system 24 h after LPS exposure that we observed with microglia conditioned medium in primary astrocytes [21].

The mechanism behind the increase in the Nrf2 system 24 h after LPS administration could be related to activation of certain kinases. We observed previously that inhibition of ERK1/2, Akt and JNK reduced Nrf2 mediated transcription in astrocytes [17,21]. This is in accord with a recent report showing that LPS stimulated expression of HO-1, a down-stream product of Nrf2 transcription, involved Akt and ERK1/2 activation [23]. In the present study the levels of phosphorylated/activated Akt were increased 24 h after LPS exposure (JNK was below detection limit and ERK1/2 too variable). It is thus possible that the observed protection against hypoxic-ischemic injury 24 h after LPS treatment is partly mediated via activation of Akt, in accordance with earlier studies on hypoxic preconditioning *in vivo* [24]. In parallel with the activation of the Nrf2 system 24 h after LPS exposure we observed a profound down-regulation of the sequester protein Keap1. Similar down-regulation of Keap1 and up-regulation of Nrf2 has also been observed in the brain after MCA occlusion in

adult mice [12]. The reason for the opposite effects on Nrf2 and Keap1 levels might be that activation of Nrf2 shifts the ubiqutination (and degradation) from Nrf2 to Keap1 [25].

One additional mechanism that may be involved in the observed positive effects on the Nrf2 system is the acetylation of proteins, i.e. histones, transcription factors or both. We recently showed that down-regulation of the Nrf2 system by medium from LPS (10 ng/mL) activated microglia was parallel to decreased acetylation of H3 and elevated trimethylation of H3-K9. These effects of microglia conditioned medium on both the Nrf2 system and the histone acetylation levels were reversed by HDAC inhibitors [17]. Here activation of the Nrf2 system was accompanied by elevated acetylation of H4 and increased tri-methylation of H3. On the basis of a study showing that TNFα and H_2O_2 elevate H4 histone acetylation *in vitro* [26] we hypothesize that the elevated acetylation level of H4 *in vivo* may be related to TNFα or oxidative stress induced by systemic LPS treatment.

The levels of H3-K9 trimethylation was elevated 24 h after systemic LPS but restored to control levels after 72 h. Trimethylation of H3-K9 is generally associated with repression of transcription and dimethylation of H3-K9 with binding of DNA-methylases that can cause more persistent epigenetic modification of CpG islands in various promoters [27]. These effects of H3-K9 may thus underlie the down-regulation of genes in the rat brain after systemic administration of LPS [16]. However, it should be noted that H3-K9 trimethylation in transcribed regions has been correlated with active gene transcription rather than transcriptional repression [28]. Obviously, to confirm the potential significance of the H3-K9 trimethylation on Nrf2/PGC-1α mediated transcription further more detailed studies are needed.

The increased acetylation levels of H4 imply elevated activity of histone acetyl transferases (HATs), which implicate that also Nrf2 is acetylated. Elevated acetylation of Nrf2 by HATs such as CBP increases transcription mediated by Nrf2 which could be one mechanism behind the upregulation of the Nrf2 system observed in our study [29]. The apparent elevated HAT activity could be related to the increase in Akt activation as nuclear Akt interacts with and phosphorylates p300 in response to TNFα [30].

Upregulation of MnSOD after LPS treatment in adult rats has been observed earlier *in vivo* and *in vitro* studies [20]. In that study LPS administration reduced infarct area when given 72 h prior to MCA occlusion [20]. The levels of MnSOD were measured 12 and 72 h following LPS but were only increased after 72 h. In our case we observed a significant increase in MnSOD after 24 h but no increase at 72 h after LPS. The apparent discrepancy between the results most certainly relates to the differences in models, i.e. we used 7-day old rats and 0.3 mg LPS/kg whereas Bordet and co-workers employed adult rats and 0.5 mg LPS/kg. TNFα was suggested to mediate the protective effects of LPS treatment and the increase in MnSOD [20].

After 72 h the effect of systemic LPS administration on the Nrf2 system was more or less reversed in the present study, i.e. Nrf2, γGCL-M and γGCL-C levels were down-regulated whereas Keap1 levels were higher compared to saline treated controls. This is in most

respects similar to our results *in vitro* on the effects of microglia conditioned medium on the Nrf2 system in astrocyte cultures [17,21].

We have earlier evaluated the role of kinases in the up- and down-regulation of the Nrf2 system [17,21]. These studies *in vitro* showed that microglia conditioned medium exert negative effects on the Nrf2 system via p38 MAPK and GSK3β activation whereas positive effects were observed for activation of JNK, ERK, Akt/PI3 kinase. Here we analysed activation of p38 and Akt (phosphorylated GSK3β and JNK were below detection limit and ERK1/2 showed too high variation). The level of phosphorylated p38 MAPK was upregulated at 72 h after LPS administration, in accordance with our previous *in vitro* studies showing that when the Nrf2 system was down-regulated, p38 MAPK was phosphorylated. The elevated level of p38 MAPK can probably explain the decreased level of Nrf2 in the nucleus. Genetic deletion and pharmacological inhibition of p38 MAPK have earlier been shown to increase export of p38 MAPK from the nucleus and to decrease Nrf2 mediated transcription of phase II genes [31,32].

As noted above we have shown that medium from 72 h LPS (10 ng/mL) activated microglia down-regulated the Nrf2 system in astrocytes, which occurred in parallel to decreased acetylation of H3, H4, elevated activity of HDACs and increased tri-methylation of H3-K9. The effects on both the Nrf2 system and the histone acetylation levels were reversed by HDAC inhibitors, which also protected against oxidative stress induced cell damage. Interestingly, the deacetylation was obliterated by inhibitors of p38 MAPK and GSK3β [17]. Here we did not observe any significant changes in H3 acetylation after 72 h although a trend towards decreased acetylation was observed. The data show clearly that between 24 and 72 h the acetylation level of H4 is decreased, meaning that either the H4 acetylation by HATs is decreased or that the high acetylation level at 24 h is decreased by elevated HDAC activity. This fits well with our *in vitro* results where we showed that after 72 h but not after 24 h microglia conditioned medium caused elevated HDAC activity in astrocytes [17].

In summary, systemic LPS in neonatal rats induced time dependent changes in Nrf2 and PGC-1α that correlated well with the protective effect after 24 h and the deleterious effects after 72 h of systemic LPS reported earlier [15,16]. Thus, up-regulations of Nrf2, the modulatory and catalytic subunits of γGCL as well as PGC-1α and MnSOD were observed at 24 h after LPS treatment whereas Nrf2 and γGCL subunits were down-regulated and, PGC-1α and MnSOD were normalised at 72 h following LPS treatment. The positive effects on the inducible anti-oxidant defense after 24 h correlated with activated Akt and increased acetylation of H3 whereas the negative effect after 72 h was in parallel with elevated p38 MAPK activation and normalised Akt and H3 acetylation.

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Abbreviations

γGCL	gamma-glutamylcysteine ligase
γGCL-C	gamma-glutamylcysteine ligase catalytic subunit
γGCL-M	gamma-glutamylcysteine ligase modulatory subunit
GSH	glutathione
GSK3β	glycogen synthase kinase-3 beta
GSH	glutathione
Keap1	Kelch-like ECH-associated protein 1
ERK1/2	extracellular regulated kinase
JNK	c-Jun N-terminal kinase
H3-K9	lysine 9 in histone-3
LPS	lipopolysaccaride
MAPKs	mitogen-activated protein kinases
MCM	microglia-conditioned medium
MEK	mitogen-activated protein kinase kinase
MnSOD	manganese-superoxide dismutase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	nuclear factor-erythroid 2-related factor 2
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
***t*BHQ**	tertbutylhydroquinone
TNFα	tumor necrosis factor-alpha

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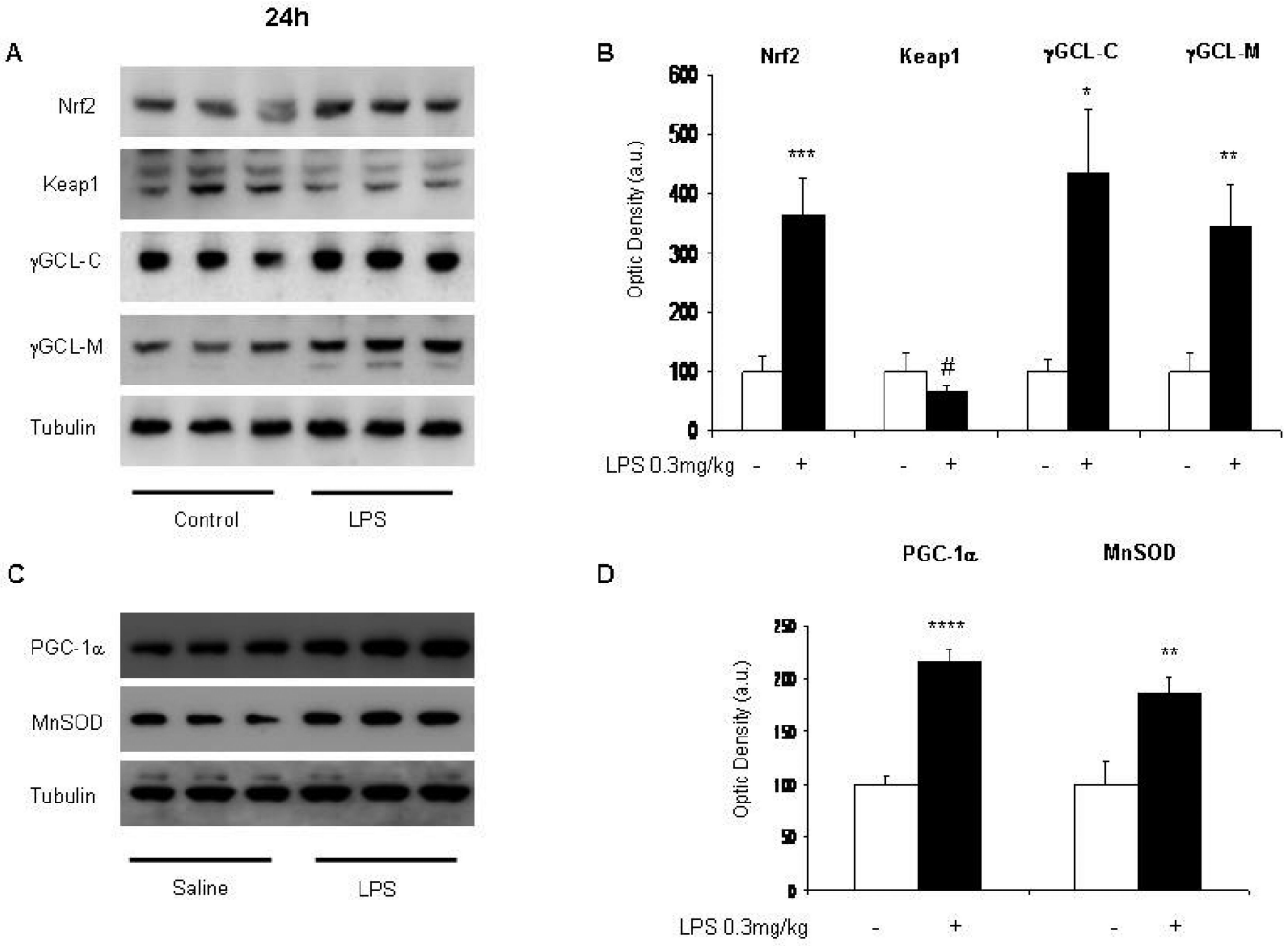

Fig. 1.

Systemic LPS (0.3 mg/kg i.p.) induced the antioxidant defence systems after 24 h. Seven-day old rat pups were exposed to either saline (n = 9) or LPS (0.3 mg/kg) (n = 11) for 24 h after which their brains were removed for the protein expression analysis of Nrf2, Keap1, γGCL-C and γGCL-M subunits was analysed (A). In (B), the densitometric analysis of western blot is shown. Statistics: *p<0.05 vs saline; **p<0.01 vs saline; ***p<0.005 vs saline; #p<0.05 vs saline (Mann-Whitman U test). A similar analysis was performed for the protein expression of PGC-1α and MnSOD (C). In (D), the densitometric analysis shows: ****p<0.001 vs saline, **p<0.01 vs saline. Results are expressed as mean ± SEM.

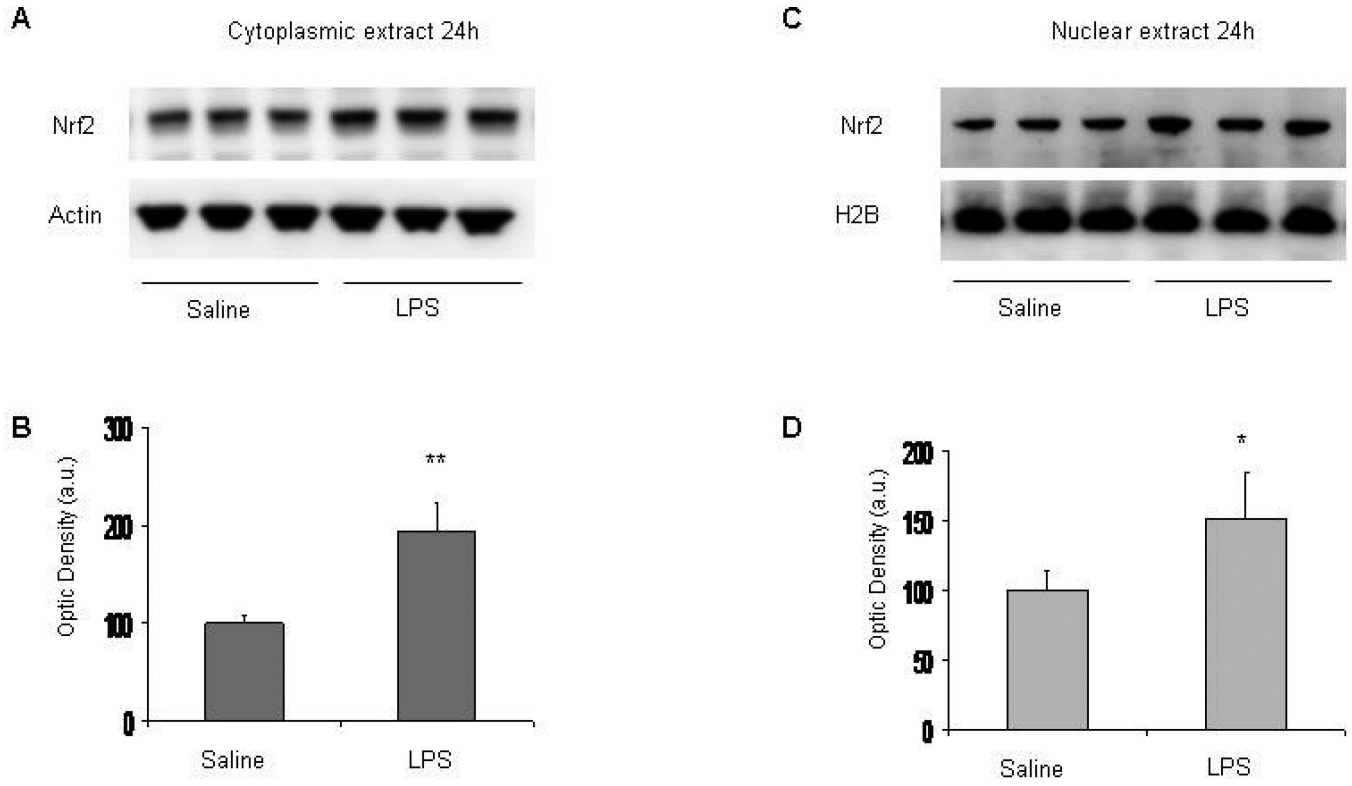

Fig. 2.

Nuclear and cytosolic extracts from brain homogenates of rats injected with LPS (0.3 mg/kg i.p.) showed increased Nrf2 protein levels after 24 h. (A) The Nrf2 protein level in cytoplasmic extract of brains of 7-day old rats 24 h after injection (i.p.) of either saline or LPS (0.3 g/kg). The densitometric analysis of the western blots is shown in (B). Statistics: **$p<0.01$ vs saline. (C) A similar analysis was performed for the protein expression of Nrf2 in the nuclear fraction. In (D), the densitometric analysis shows: *$p<0.05$ vs saline. Results are expressed as mean ± SEM.

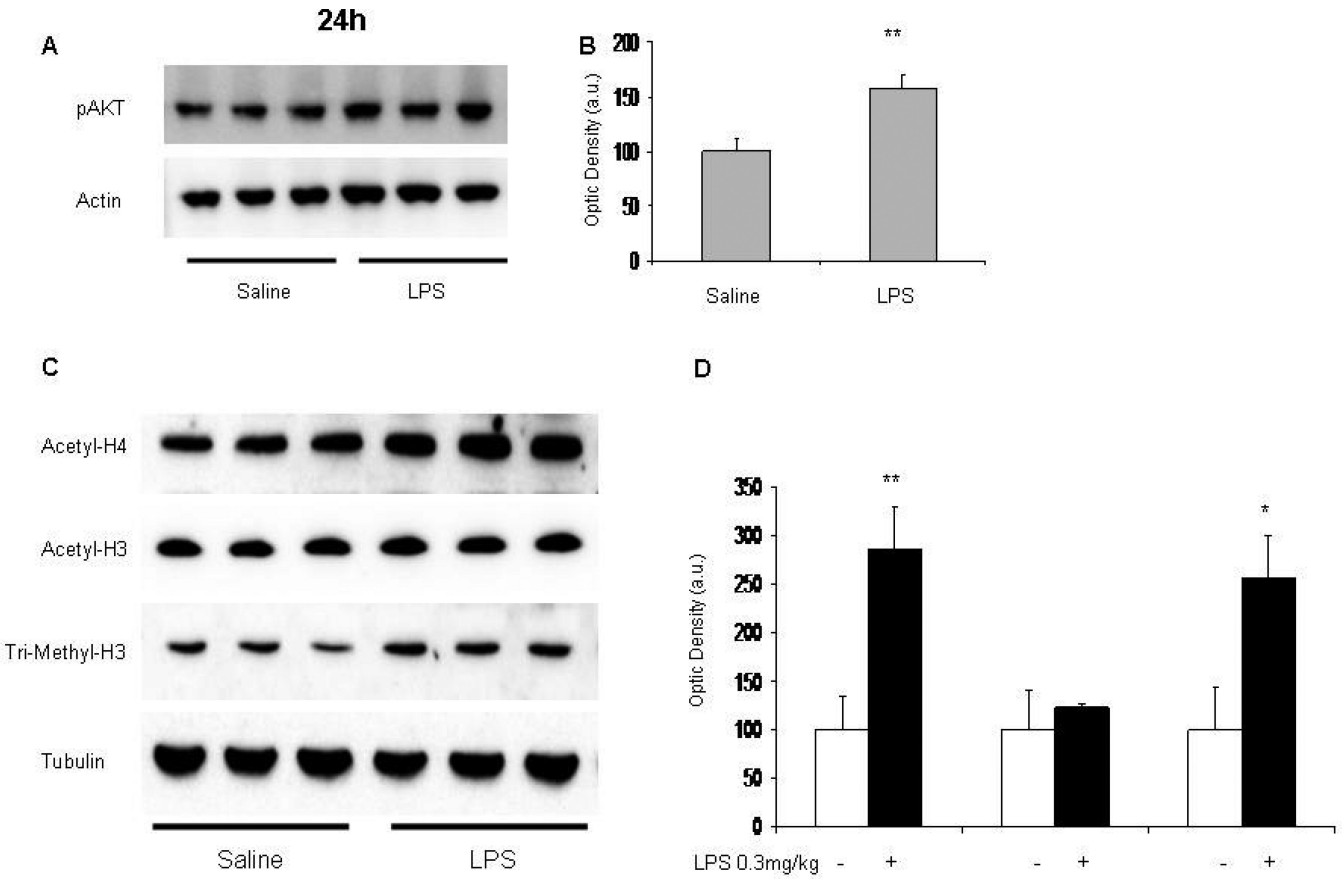

Fig. 3.

(A) Systemic LPS (0.3 mg/kg i.p.) increased the level of phosphorylated Akt after 24 h. The densitometric analysis of western blot is shown in (B). Statistics: **p<0.01 vs saline. (C) Exposure for 24 h to LPS (0.3 mg/kg) i.p. modified the acetylation/methylation pattern of histones H4 and H3. In (D), the densitometric analysis shows: *p<0.05 vs saline. Results are expressed as mean ± SEM.

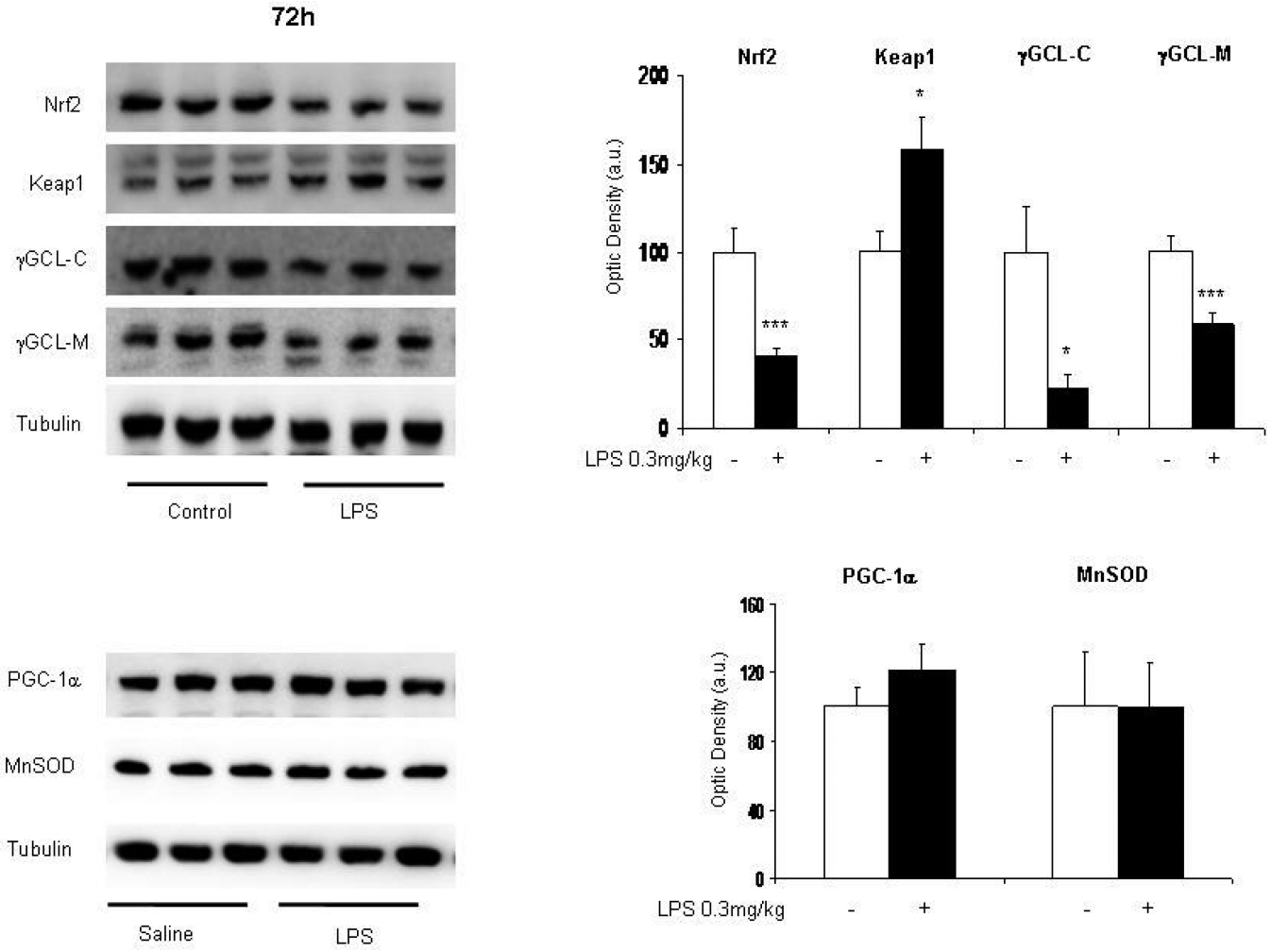

Fig. 4.

Systemic LPS (0.3 mg/kg i.p.) caused decreased antioxidant defence levels after 72 h. (A) Seven-day old rat pups were injected with saline (n = 11) or LPS (0.3 mg/kg) (n = 9) and after 72 h their brains were removed for protein level analysis of Nrf2, Keap1, γGCL-C and γGCL-M subunits. The densitometric analysis of western blot is shown in (B). Statistics: *p<0.05 vs saline; ***p<0.005 vs saline. (C) A similar analysis was performed for the protein expression of PGC-1α and MnSOD. No changes were observed in these protein levels. The densitometric analysis is shown in (D). Results are expressed as mean ± SEM.

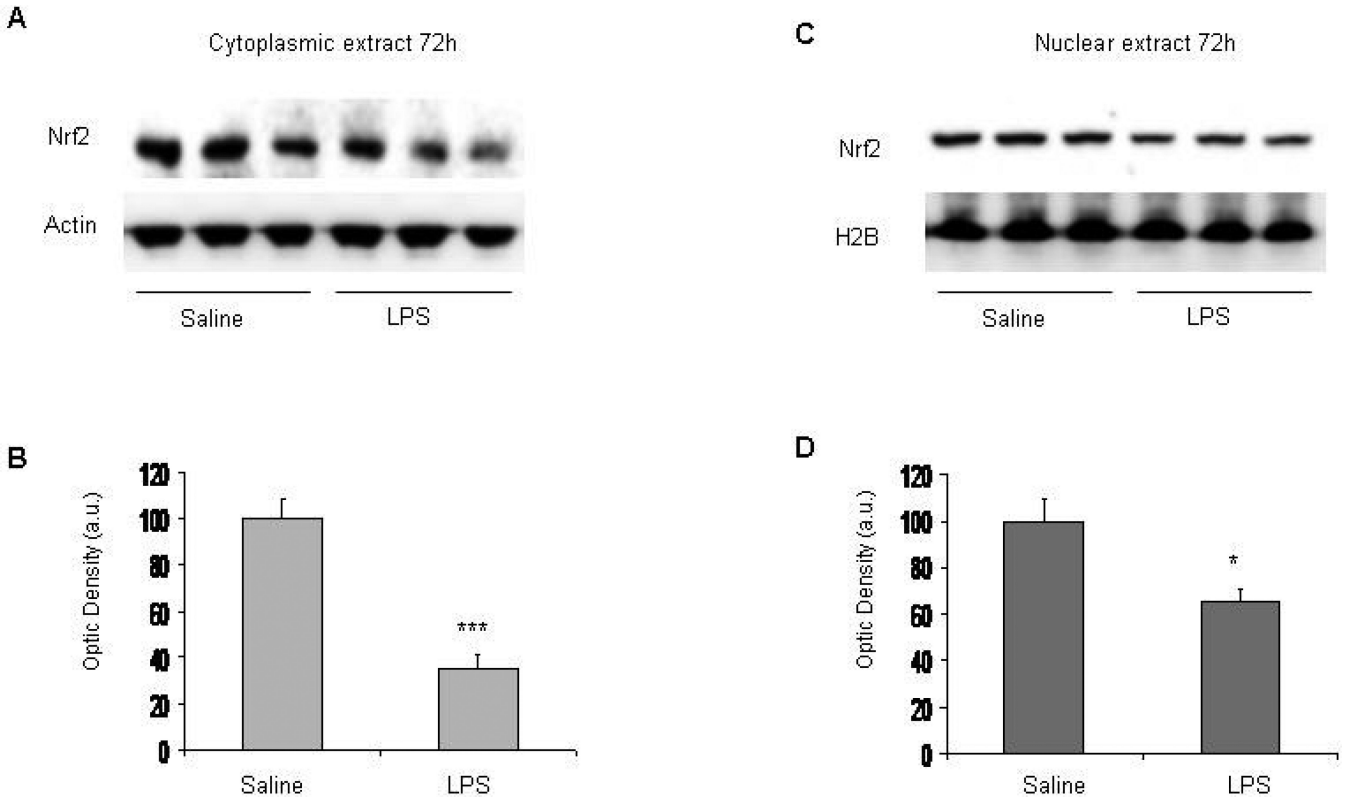

Fig. 5.

Nuclear and cytosolic extracts from brain homogenates of rats injected with LPS (0.3 mg/kg i.p.) showed decreased levels of Nrf2 protein levels after 72 h. (A) The levels of Nrf2 protein in the cytosolic fraction of brain extracts 72 h after injection of saline or LPS (0.3 mg/kg) in 7-day old rats. The densitometric analysis of western blot is shown in (B). Statistics: ***$p<0.005$ vs saline. (C) A similar analysis was performed for the protein expression of Nrf2 in the nuclear fraction. The densitometric analysis is shown in (D). Statistics: *$p<0.05$ vs saline. Results are expressed as mean ± SEM.

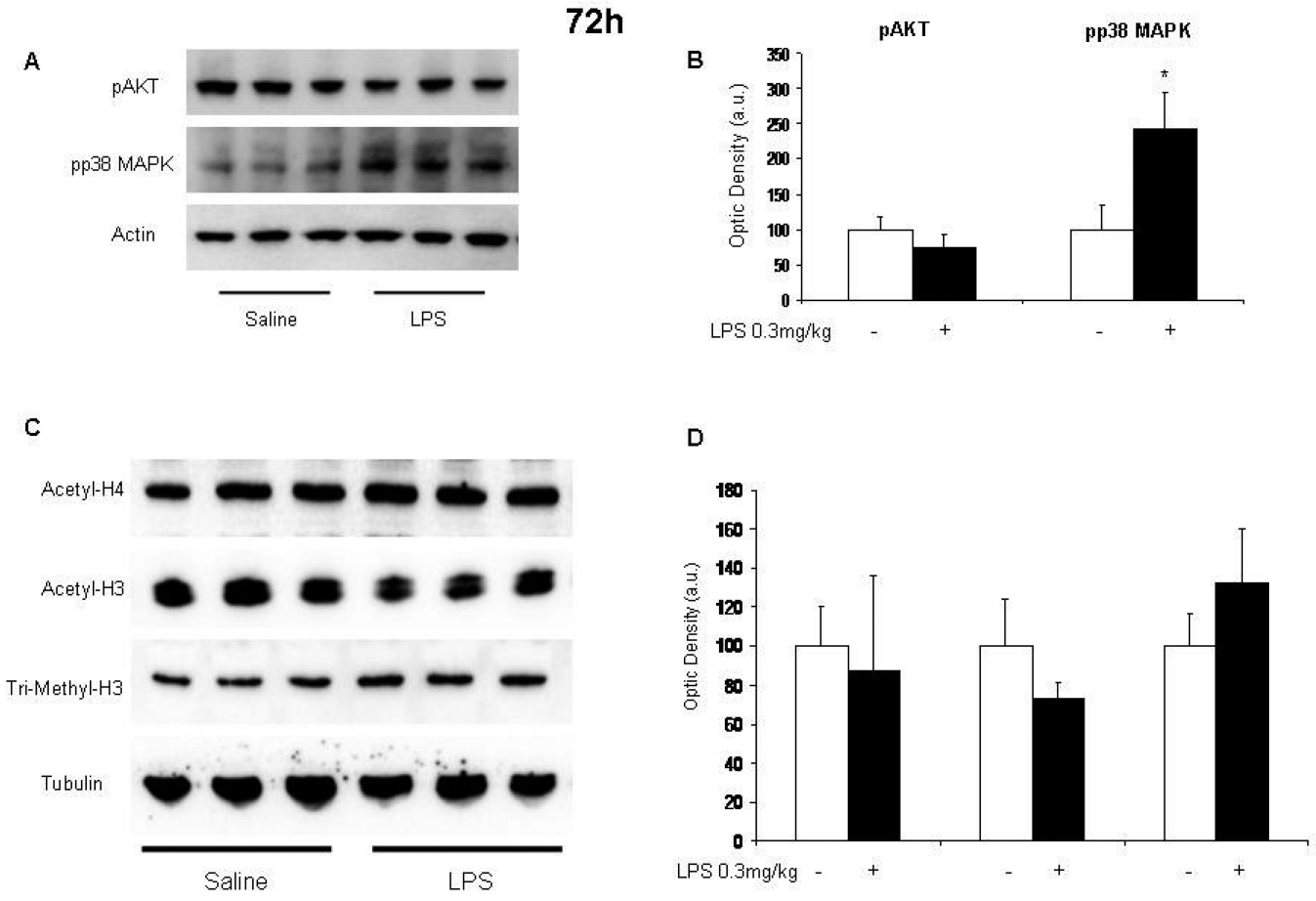

Fig. 6.

(A) Systemic LPS (0.3 mg/kg i.p.) activated p38 MAPK. In (B), the densitometric analysis of western blot is shown. Statistics: *$p<0.05$ vs saline. (C) The acetylation pattern of Histone H3, showed a tendency (although without statistical significance) to be down-regulated 72 h following systemic LPS (0.3 mg/kg i.p.). In (D), the densitometric analysis is shown. Results are expressed as mean ± SEM.