RESEARCH ARTICLE

Anti-cytokine and anti-hyperalgesic effects of aliskiren in experimental models of inflammation

Rakesh B. Patel*, Kanaiyalal D. Prajapati, Bhavin M. Sonara, Manoranjan M. Sharma, Vishwanath D. Pawar, Mukul R. Jain

Department of Pharmacology, Zydus Research Centre, Sarkhej-Bavla National Highway-8A, Moraiya, Ahmedabad 382 213, Gujarat, India.

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ABSTRACT

The renin-angiotensin system is involved in pathogenesis and progression of inflammatory responses. In present study, we investigated the anti-cytokine and anti-hyperalgesic potential of aliskiren in LPS (lipopolysaccharide) and CFA (*complete* Freund's *adjuvant)* induced inflammation in rodents. After aliskiren treatment, plasma cytokines (TNF-α, IL-6) and plasma renin activity (PRA) were estimated in LPS treated mice. In CFA induced inflammatory pain model, rats were treated with aliskiren and thermal hyperalgesia was evaluated and TNF-α and IL-6 were estimated in paw. In LPS treated ICR mice, elevated TNF-α and IL-6 levels and PRA were attenuated by aliskiren. In LPS treated double transgenic mice (dTG), harboring human renin and human angiotensinogen and C57BL/6J mice, elevated TNF-α level was inhibited by aliskiren. Moreover after LPS treatment, TNF-α level was high (2.16 fold) in dTG mice than C57BL/6J. These data indicates that renin overexpression system in dTG mice is responsible for higher TNF-α level suggests role of renin in inflammation. Moreover, Aliskiren showed anti-hyperalgesic activity in CFA induced inflammatory hyperalgesia in rats, which might be due to inhibition TNF-α and IL-6 levels, suggests the role of local renin in inflammation. Our results indicate the role of renin in experimental inflammation which is inhibited by aliskiren.

Key words: Aliskiren; renin; CFA; LPS; inflammation; cytokine

INTRODUCTION

The involvement of the renin-angiotensin system (RAS) in pathogenesis and progression of inflammatory responses has received much attention recently. Angiotensin II (Ang II), the main peptide of the RAS, is considered as a pro-inflammatory mediator that participates in the inflammatory response in several pathological processes.[1-3] It modulates the production of adhesion molecules, cytokines (IL-6 and TNF-α), chemokines and the responses of immune and inflammatory cells, such as chemotaxis, proliferation and differentiation.[4-6] Moreover, renin upregulates cytokines production acting on pro-renin receptor and independent of angiotensin II.[7] In double transgenic (dTG) (harboring human renin and human angiotensinogen, R+/A+) mice, there was overexpression of human renin and human angiotensinogen, showed high levels of plasma Ang-II.[8]

Aliskiren inhibits the renin angiotensin system by directly inhibiting renin and subsequently decreases the production of angiotensin II. Aliskiren has been found to be beneficial in hypertension, chronic kidney disease, cardiac hypertrophy, atherosclerosis and vascular inflammation.[9-11] . Various studies have also shown anti-inflammatory and antioxidant activities of aliskiren.[9;12-15] Moreover, in our previous study we demonstrated analgesic activity of aliskiren in various pain models.[16] Furthermore, in our previous study, analgesic activity of aliskiren was attributed to anti-cytokine activity in formalin induced pain model.[16]

Based on mentioned evidences we decided to investigate the role of renin in LPS induced inflammation in ICR, C57 and double transgenic (dTG) (harboring human renin and human angiotensinogen, R+/A+) mice and inflammatory pain in rats. Therefore, the present study was designed to explore the anti-cytokine and anti-hyperalgesic potential of aliskiren in experimental models of inflammation.

Address for correspondence

Mr. Rakesh B Patel
Department of Pharmacology,
Zydus Research Centre,
Ahmedabad 382 213, Gujarat, India.
E-mail:rakepatel@gmail.com

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MATERIALS AND METHODS

Animals

Eight to ten weeks old of healthy ICR, C57BL/6 and double transgenic (dTG)(harboring human renin and human angiotensinogen, R+/A+) mice (21-25 g) and Sprague Dawley rats (190-250 g) of either sex were employed in the study. Mice harboring human renin (R+) and human angiotensinogen (A+) were procured from Jackson laboratory, USA. Double transgenic (dTG) (harboring human renin and human angiotensinogen, R+/A+) mice inhouse bred at Zydus Research Center by using R+ and A+ strains. Mice (ICR, C57BL/6) and rats were bred in-house at Zydus Research Center, Ahmedabad, India and housed in individually ventilated cages under controlled temperature (18-25°C), humidity (30-70 % RH) and normal light/dark (12h/12h) cycle conditions. Food and water were provided ad libitum. Institutional Animal Ethics Committee (IAEC) approved experimental protocol and animal care was taken as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. At the end of experiments, all animals were euthanized by CO_2.

Drugs and Chemicals

Aliskiren was (Zhejiang Apeloa Jiayuan Pharma. Ltd., China) dissolved in normal saline (HPLC purity of aliskiren: 99.40%). Lipopolysaccharide (Sigma, USA) and tramadol (Cadila Healthcare Ltd, Ahmedabad) was dissolved in normal saline. TNF-α and IL-6 ELISA kits (BD OptEIA) were procured from BD Biosciences, USA. PRA (plasma renin activity) kits were procured from Diasorin, Inc., USA. CFA (*complete* Freund's *adjuvant)* was procured from Sigma, USA. Analytical grade chemicals/reagents were used in the study.

Effect of aliskiren on Lipopolysaccharide (LPS) induced cytokines (TNF-α and IL-6) levels.

ICR mice were treated with vehicle, aliskiren (3, 10 and 30 mg/kg, i.p) thirty min before LPS (10 µg/mouse, i.p.) treatment and naïve mice were injected with the equivalent volume of normal saline. Blood was collected by retro-orbital puncture under isoflurane anaesthesia at 2 hrs after LPS treatment in EDTA containing tubes for cytokines estimation. Plasma was separated by centrifugation at 2000 *g* for 15 min at 4ºC and stored at-80 ºC until analysis. TNF-α and IL-6 were estimated in plasma by enzyme-linked immuno-sorbent assay (ELISA) kits according to the manufacturer's instructions (BD Biosciences, USA). In C57BL/6 and double transgenic (R+/A+) mice, aliskiren (10 mg/kg, i.p.) was injected thirty min before LPS (10 µg/mouse, i.p.) treatment and TNF-α was measured in plasma as mentioned above.

Effect of aliskiren on LPS induced PRA

ICR mice were treated with vehicle or aliskiren (3, 10 and 30 mg/kg, i.p) thirty min before LPS (10 µg/mouse, i.p.) treatment and naïve mice were injected with the equivalent volume of normal saline. Blood was collected by retro-orbital puncture under isoflurane anaesthesia at 2hrs after LPS treatment in EDTA containing tubes for PRA estimation. PRA was estimated by radioimmunoassay (RIA) kits according to the manufacturer's instructions (Diasorin, Inc., USA).

PRA was expressed as Angiotensin-I (ng/ml/hr) generation.

Effect of Aliskiren on CFA induced thermal hyperalgesia in rats

Rats were treated with CFA (100 µg/rat, i.pl.). Rats were evaluated for thermal hyperalgesia 24 hrs after CFA treatment. Analgesiometer (IITC Life Sciences, USA) was used to measure paw withdrawal latency. The light beam was focused on the ipsilateral paw and paw withdrawal latency was recorded at active intensity (Active Intensity: 35 %). The cut-off time was fixed at 20 sec to avoid paw damage. Paw withdrawal latency was recorded at 0 and 60 min after vehicle, tramadol (30 mg/kg, i.p.) and aliskiren (25, 50 and 75 mg/kg, i.p.) treatment. Paw withdrawal latency was also recorded in naïve rats. Maximum possible effect (MPE), which represents anti-

hyperalgesic activity, was calculated using following formula:

$$\% \text{ MPE} = 100*\frac{\{(\text{Posttreatment latency} - \text{Pretreatment latency}\}}{(\text{Cut off time} - \text{Pretreatment latency})}$$

After completion of experiment, animals were sacrificed by using CO_2 and right hind paws were collected in dry ice. Paw was homogenized in phosphate buffer saline buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and homogenates were centrifuged at 5000 g at 4ºC for 15 min. Supernatants were stored at -80 ºC until analysis. TNF-α and IL-6 were estimated in supernatants using ELISA kits. Protein was estimated in supernatants by method described by Lowry et al[17].

Statistical analysis

Data were expressed as mean ± SEM. Data were analysed by one way ANOVA (analysis of variance) followed by Dunnett's posttest. The $P < 0.05$ was considered statistically significant.

RESULTS

Effect of aliskiren on LPS induced cytokines (TNF-α and IL-6) levels in ICR mice

In ICR mice, TNF-α levels, IL-6 levels and PRA were elevated which were significantly attenuated dose dependently by aliskiren treatment (3, 10 and 30 mg/kg, i.p) (Fig. 1. and 2.).The levels of TNF-α (2.99 ± 2.52 pg/ml) and IL-6 (5.25 ± 2.98 pg/ml) were very less in mice (naïve) not receiving LPS (Fig. 1. and 2.).

Effect of aliskiren on LPS induced PRA in ICR mice

In ICR mice, after LPS treatment, PRA was increased as compared to mice receiving vehicle of aliskiren (Fig. 3.). Aliskiren treatment (3, 10 and 30 mg/kg, i.p) dose dependently reduced increased PRA in ICR mice (Fig. 3.).

Effect of aliskiren on LPS induced TNF-α level in C57BL/6 and double transgenic (R+/A+) mice

In dTG and C57BL/6J, (background strain for dTG) mice after LPS treatment, TNF-α level were enhanced. Moreover after LPS treatment, TNF-α levels were high (2.16 fold) in dTG mice than C57BL/6J (Fig. 4.). In both mice strains, elevated TNF-α level was inhibited by 10 mg/kg, i.p aliskiren (Fig. 4.). The levels of TNF-α (6.29 ± 3.22 pg/ml in dTG, 3.29 ± 2.36 pg/ml in C57BL/6J) were very less in (naïve) mice not receiving LPS (Fig. 4.).

Effect of aliskiren on CFA induced thermal hyperalgesia in rats

Aliskiren produced dose dependent attenuation of CFA induced thermal hyperalgesia (Fig. 5.).

Tramadol (30 mg/kg, i.p.) which was taken as positive control, also showed significant anti-hyperalgesic activity as compared to vehicle (Fig. 5.).

CFA injection in paw caused significant increase in local TNF-α level (22.80 ± 1.46 pg/mg of protein) as compared to naive animals (5.45 ± 0.49 pg/mg of protein). In CFA-injected rats, aliskiren treatment (50 and 75 mg/kg, i.p.) produced dose-dependent reduction in TNF-α (14.61 ± 0.78 and 8.61 ± 0.96 pg/mg of protein) levels in paw homogenate (Table 1) as compared to vehicle treatment.

Similarly, CFA injection in paw caused significant increase in local IL-6 level (19.82 ± 1.02 pg/mg of protein) as compared naive animal (56.68 ± 3.95 pg/mg of protein). Aliskiren (50 and 75 mg/kg, i.p.) dose dependently reduced the IL-6 levels (38.78 ± 2.112 and 29.82 ± 3.41 pg/mg of protein) in paw (Table 1) after CFA injection as compared to vehicle treatment.

DISCUSSION

In present study, we for the first time investigated effect of aliskiren in LPS and CFA induced inflammation in rodents. We evaluated anti-cytokine activity of aliskiren in normal ICR mice. Aliskiren dose dependently suppressed LPS induced PRA, TNF-α and IL6 levels in ICR mice. Further, to evaluate role of RAS and anti-cytokine activity of aliskiren in LPS induced inflammation, RAS overexpressed (dTG) mice were used. In dTG mice, severity of inflammation (cytokines levels) is more as compared to normal mice, which indicates role of RAS in LPS induced inflammation. Aliskiren, a direct inhibitor of renin, attenuated

cytokines levels in LPS induced inflammation in dTG mice. It is reported that CFA induced hyperalgesia in rats enhances production of tissue cytokines.[18-20] In our results, aliskiren has antihyperalgesic activity in CFA induced hyperalgesia in rats that might be due to inhibition of TNF-α and IL6 levels in rat paw. In rats the dose of aliskiren was higher than those used in mice due to species specific differences in potency (IC_{50}) for renin inhibition in rat (80 nM) against mice (6.2 nM).[10] Reported data showed that aliskiren (25 mg/kg, ip & 50 mg/kg, sc) for 4 weeks treatment had slight effect blood pressure in normal rodents.[10;21] So in present study, selected doses of aliskiren may not have hypotensive effect in normal rodents.

LPS induces an innate immune response with a major upregulation of pro-inflammatory factors including enhanced pro-inflammatory cytokines production.[22] In our study, TNF-α IL-6 and PRA were elevated in ICR mice, which were significantly attenuated dose dependently by aliskiren treatment (3, 10 and 30 mg/kg, i.p). Moreover, in double transgenic (R+/A+) mice, LPS administration significantly increased (2.16 fold) cytokine (TNF-α) level as compared to C57BL/6J mice, indicating role of RAS in activation of proinflammatory cytokines. In double transgenic (R+/A+) mice, there was overexpression of human renin and human angiotensinogen, showed high levels of plasma Ang-II.[8] ANG II and LPS promote inflammation; activating similar transcriptional and posttranscriptional mechanisms interrelated in complex intracellular cross-talk mechanisms. These include *1*) activation of phospholipase C and protein kinase C, leading to NAD(P)H oxidase stimulation and enhanced reactive oxygen species (ROS) formation; *2*) activation of transcription factors, such as NF-κB, and production of proinflammatory cytokines; *3*) cyclooxygenase-2 (COX-2) activation with enhanced proinflammatory prostaglandin E_2 (PGE_2) formation; and *4*) inducible nitric oxide synthase (iNOS) induction with increased nitric oxide production.[23-27] So aliskiren by inhibiting renin, subsequently Ang-II production, might be inhibited LPS induced inflammation.

It can be concluded that overexpressed RAS in dTG mice might contributes to higher LPS induced TNF-α levels. Cytokines are important mediators of inflammation and elevated levels of pro-inflammatory cytokines are observed in the inflammatory conditions in human and animals.[28-31] Anti-inflammatory and anti-cytokines activity of aliskiren is well reported in various studies.[12-15] Our results are in agreement with our previous work, in which anti-cytokine activity of aliskiren is reported in formalin-induced pain.[16] Together, these data supports anti-cytokine activity of aliskiren in LPS induced inflammation by inhibiting renin.

In our study, aliskiren showed anti-cytokine and anti-hyperalgesic activity dose dependently in CFA induced hyperalgesia and paw cytokines (TNF- α and IL-6) level. It is reported that proinflammatory cytokines like TNF-α and interleukin-1 beta (IL-1β) induce and facilitate neuropathic as well as inflammatory pain.[32-34] Further, it has been shown that CFA injection resulted in a significant elevation of TNF-α and IL-1β gene expression in the inflamed plantar skin, which is in accordance with the change of TNF-α and IL-1β at the protein level in the same tissue.[32-34] Pro-inflammatory cytokines, such as TNF-α and IL-1β, reduced thermal or mechanical pain thresholds upon intraplantar application.[32;33] Moreover, renin upregulates cytokines production acting on pro-renin receptor and independent of angiotensin II.[7] There are few reports that have shown beneficial effects of aliskiren by direct inhibition of pro-renin receptors and an anti-inflammatory effect independent of angiotensin II.[35;36] Pro-inflammatory cytokine antagonists were further able to reduce hyperalgesia in inflammation models, indicating that the activation of pro-inflammatory cytokines is an important step in the generation of inflammatory pain.[37] Our results demonstrate that the aliskiren may have anti-inflammatory effect to suppress CFA induced hyperalgesia. This hypothesis is supported by our

observation that aliskiren reduced TNF-α and IL-6 levels after CFA injection in rat paw. Our observations are corroborated by other reports indicating anti-inflammatory potential of aliskiren.[9;12;13] Therefore, aliskiren by inhibiting renin may inhibit cytokines and produce antihyperalgesic activity. However further study is required for molecular mechanism for role of renin in LPS and CFA induced inflammation.

CONCLUSION

In conclusion, our study demonstrates that blockade of renin ameliorates inflammation, reaffirming the critical role of the RAS in the inflammation. The protective actions of aliskiren might be dependent on both the Ang II-dependent pathway and the (pro) renin receptor-mediated pathway. This study also suggests the anti-inflammatory and antihyperalgesic effects of aliskiren in inflammatory models, which are mediated via the inhibition of renin and pro-inflammatory mediator production.

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CONFLICT OF INTEREST

There is no conflict of interest associated with this manuscript.

Treatment	TNF-α (pg/mg of protein)	IL-6 (pg/mg of protein)
Naïve	5.45 ± 0.49	19.82 ± 1.02
CFA + Vehicle	22.80 ± 1.46+	56.68 ± 3.95+
CFA + Aliskiren (25 mg/kg, i.p.)	19.33 ± 1.17	46.81 ± 2.92
CFA + Aliskiren (50 mg/kg, i.p.)	14.61 ± 0.78*	38.78 ± 2.11*
CFA + Aliskiren (75 mg/kg, i.p.)	8.61 ± 0.96**	29.82 ± 3.41**

Table 1: Effect of aliskiren on TNF-α and IL-6 levels in CFA (100 µg/paw, i.pl.) injected rats. Data are expressed as mean ± S.E.M. for 6 rats in each group. +P< 0.01vs Naive, *P< 0.05, **P< 0.01 vs CFA + vehicle, one-way ANOVA followed by Dunnett's test.

Figure 1: Effect of aliskiren on TNF-α in LPS (10 µg/mouse, i.p.) injected mice. Data are expressed as mean ± S.E.M. for 6-7 mice in each group. *P< 0.05, ***P< 0.001 vs LPS + vehicle, one-way ANOVA followed by Dunnett's test. Values are shown above column in box are group mean.

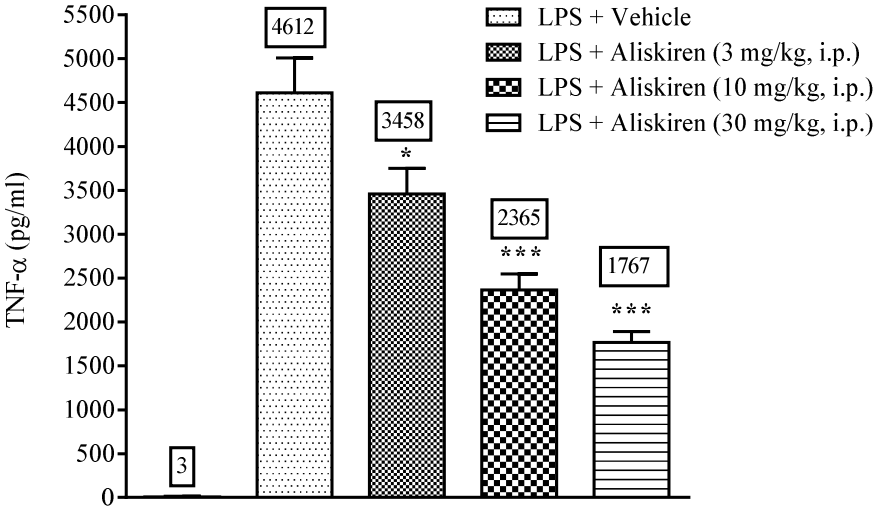


Figure 2: Effect of aliskiren on IL-6 in LPS (10 µg/mouse, i.p.) injected mice. Data are expressed as mean ± S.E.M. for 6-8 mice in each group. *P< 0.05,**P< 0.01,***P< 0.001 vs LPS + vehicle, one-way ANOVA followed by Dunnett's test. Values are shown above column in box are group mean.

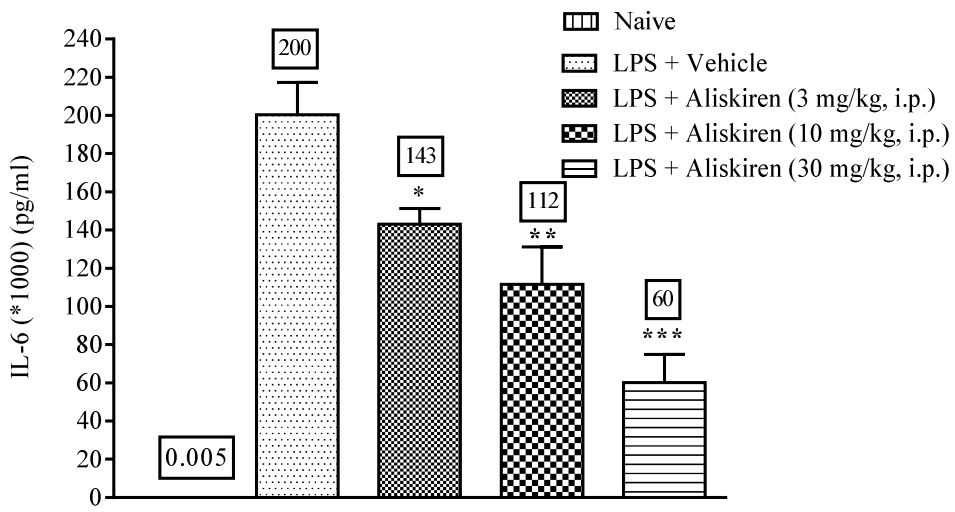


Figure 3: Effect of aliskiren on PRA in LPS (10 µg/mouse, i.p.) injected mice. Data are expressed as mean ± S.E.M. for 5 mice in each group.*** P<0.001 vs vehicle,***P< 0.001vs LPS + vehicle, one-way ANOVA followed by Dunnett's test. Values are shown above column in box are group mean.

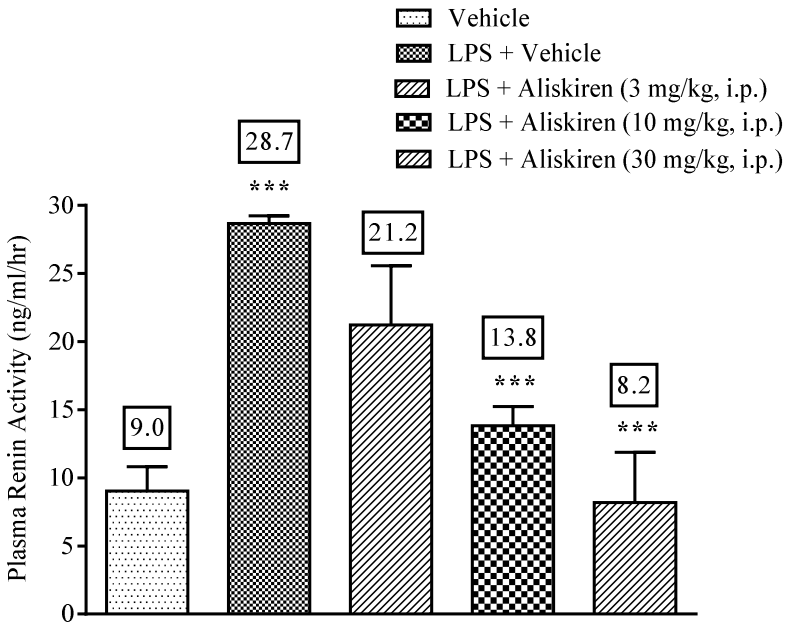


Figure 4: Effect of aliskiren on TNF-α in LPS (10 µg/mouse, i.p.) injected dTG and C57BL/6J mice. Data are expressed as mean ± S.E.M. for 6 mice in each group. *** P<0.001 vs vehicle (C57BL/6J).* P<0.05 vs vehicle (C57BL/6J), **P< 0.01 vs vehicle (dTG), one-way ANOVA followed by Dunnett's test. Values are shown above column in box are group mean.

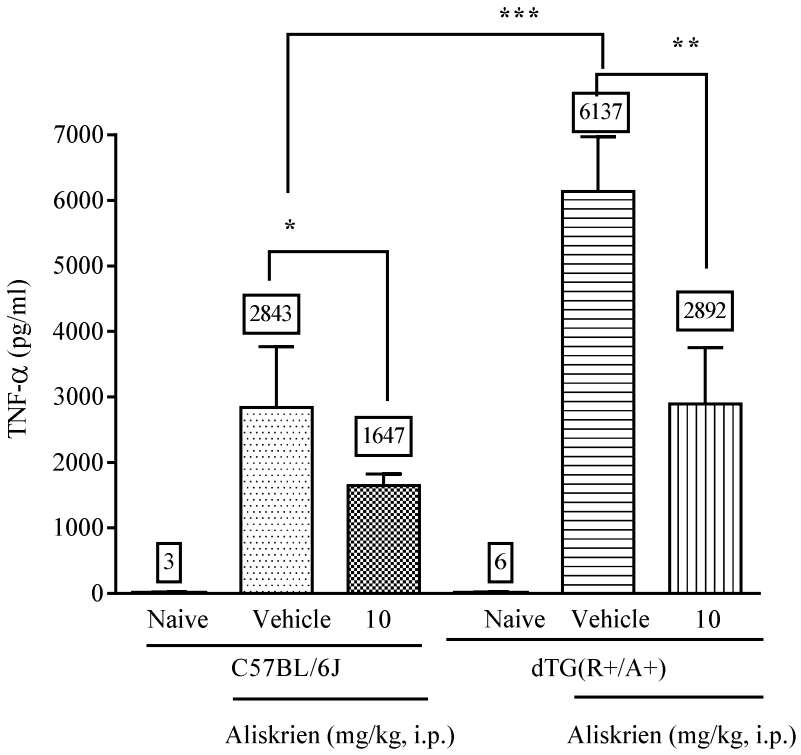
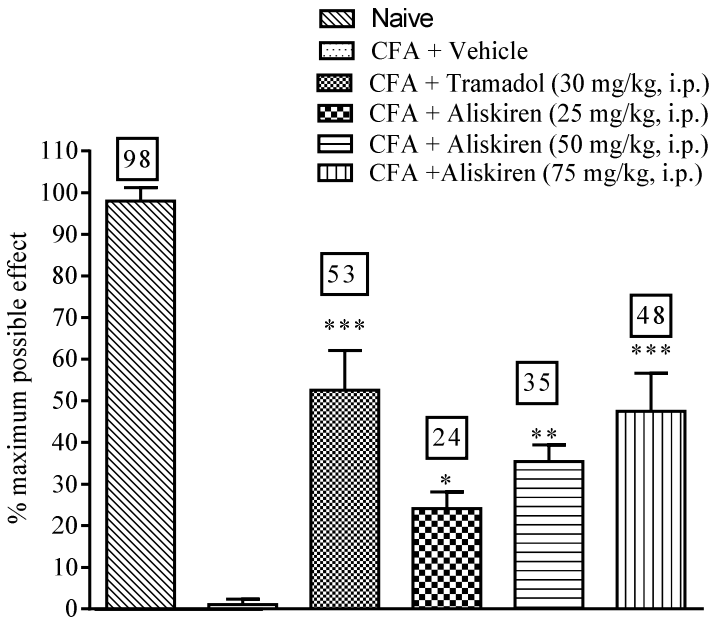


Figure 5: Effect of aliskiren on CFA induced hyperalgesia in rats. Data are expressed as mean ± S.E.M. for 6 rats in each group. *P< 0.05,*P< 0.01,***P< 0.001 vs vehicle, one-way ANOVA followed by Dunnett's test. Values are shown above column in box are group mean.



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