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Early Atorvastatin Reduces Hemorrhage after Acute Cerebral Ischemia in Diabetic Rats

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Vascular Protection with Atorvastatin after Stroke

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List of non-standard abbreviations:

GK = Goto-Kakizaki ; MCAO = middle cerebral artery occlusion ; ELISA = enzyme-linked immunosorbent assay; eNOS = endothelial nitric oxide synthase; MAP = mean arterial pressure

Abstract:

Background- Ischemic stroke is a leading cause of death in the United States and diabetes mellitus is a major risk factor for stroke. Our previous work showed that type II diabetic rats (Goto-Kakizaki - GK) have more bleeding after stroke than their normoglycemic controls (Wistar). Our aim was to evaluate the vascular protective properties of acute atorvastatin therapy after experimental ischemic stroke in diabetes and to explore the effect of stroke in GK rats compared to their normoglycemic controls. *Methods*-50 Male Wistar and 40 GK rats (270-305 g) underwent 3 hours of middle cerebral artery occlusion (MCAO) followed by reperfusion for 21 hours. Animals received atorvastatin (5mg/Kg), atorvastatin (15mg/Kg) or vehicle, administered by oral gavage, one dose 5 minutes after reperfusion and a second dose after 12 hours. At 24 hours, functional outcome was measured and brain tissue was analyzed for infarct volume, hemoglobin content and molecular biomarkers. Plasma was collected for analysis of atorvastatin concentrations. *Results*- Atorvastatin-treated groups had significantly lower bleeding rates ($p=0.0011$) and infarct volume ($p=0.0007$) compared to controls. There was a significant reduction in hemoglobin content and infarct volume only in the higher dose group (15mg/Kg) ($p<0.05$) and these benefits were more than 4 times greater in the diabetic animals. Atorvastatin (15mg/Kg) improved neurological outcome in both Wistar and GK rats ($p=0.029$) at a peak concentration of (27-77 ng/ml) and was associated with an increase in Akt phosphorylation ($p=0.0007$). *Conclusion*- Atorvastatin is a vascular protective agent after experimental ischemic stroke especially in diabetes.

Introduction

Stroke is the third leading cause of death in the United States and affects about 750,000 individuals each year(Idris et al., 2006). Type II DM is a disease that affects more than 17 million Americans. In diabetic patients, the risk of stroke is increased by 2-4 fold(Folsom et al., 1999; Idris et al., 2006; Martini and Kent, 2007). Not only is there an increase in the risk of stroke recurrence in diabetics, but these patients also have increased hospital and long-term stroke mortality and a worsening of overall stroke outcomes(Folsom et al., 1999; Idris et al., 2006). One of the contributing factors to this dramatic prognosis is that hyperglycemia is associated with intracerebral hemorrhage in acute ischemic stroke patients treated with thrombolytic agents(Martini and Kent, 2007) and this has been confirmed in animal models(Kawai et al., 1997; Quast et al., 1997). In addition, many of the detrimental pathways involved in vascular damage associated with stroke are known to be upregulated in the diabetic vasculature, including oxidative stress, endothelial dysfunction and inflammation(Fagan et al., 2005; Martini and Kent, 2007). A vascular protective agent, administered after stroke onset, may improve the safety and efficacy of thrombolytic therapy in diabetic patients. We have identified statins as likely vascular protective agents in the acute stroke period(Fagan et al., 2004) and statins are routinely prescribed for lipid lowering in these patients. Statins also have other pleiotropic effects including reduction of oxidative stress, improvement of the endothelial function, increase in the nitric oxide (NO) bioavailability and inhibition of the inflammatory and cell death mediators (Liao, 2002; Endres, 2005; Liao and Laufs, 2005). Many studies have demonstrated that statins reduce stroke risk in both primary and secondary prevention trials (4S, 1994;

WOSCOPS, 1998; CARE et al., 1999; Sever et al., 2003; CARDS et al., 2004; Amarenco et al., 2006). Statins have also been shown to be neuroprotective when administered acutely after experimental stroke (Sironi et al., 2003; Kilic et al., 2005) , yet, post-stroke effects of statins in diabetes have not been evaluated. Recent data from our group demonstrated that type II diabetic animals (Goto-Kakizaki rats (GK) are more susceptible to vascular damage after experimental cerebral ischemia than non-diabetic animals (Wistar rats)(Ergul et al., 2007) and may serve as a good model to test potential vascular protective agents after stroke in diabetes. In this study, we evaluated whether acute atorvastatin therapy is vascular protective in GK rats after experimental ischemic stroke. A set of normoglycemic rats (Wistar) was used to confirm previous reports showing post-ischemic neuroprotective effects of statins(Sironi et al., 2003; Kilic et al., 2005; Zhang et al., 2005). We also explored the effect of stroke in the diabetic GK rats compared to their normoglycemic controls.

Methods

The Institutional Animal care and Use Committee (IACUC) of the Augusta VA Medical Center approved the protocol. Male Wistar rats (n=50), from the Charles River Breeding Company (Wilmington, Massachusetts, USA) and GK rats (n=40), from Taconic farms, Inc. (Germantown, New York, USA) within a narrow range of body weight (270-305) were used.

Experimental cerebral ischemia

Anesthesia was performed by using 2% isoflurane via inhalation. Cerebral ischemia was induced using the intraluminal suture middle cerebral artery occlusion (MCAO) model(Longa et al., 1989). 19-21 mm 3-0 surgical nylon filament was introduced from

the external carotid artery lumen into the internal carotid artery to block the origin of the right MCA. The animals were kept under anesthesia for only 15 minutes for the surgical procedure. Temperature was maintained at 37.0-38.0 C all the time using a controlled heating system. The suture was removed after 3 hours of occlusion and the animals were returned to their cages. At reperfusion, animals received atorvastatin (5 mg/Kg) (Pfizer Inc., New York, NY, USA), atorvastatin (15 mg/Kg) or methyl cellulose (MC) (0.5%) (Sigma Chemical Co., St. Louis, Missouri, USA), administered twice daily by oral gavage, the first dose 5 minutes after reperfusion and the second dose after 12 hours. Atorvastatin doses chosen were previously shown by others to be neuroprotective(Hayashi et al., 2004; Yrjanheikki et al., 2005).

Physiological monitoring

Blood glucose level and body weight were measured in all animals before MCAO and before sacrifice. We also measured hemoglobin A1c at baseline using the A1c+ kit (Metrika, Sunnyvale, California, USA) in a group of 12 rats. In a fourth group (n=17), cerebral perfusion was measured using Periscan PIM 3 System (Stockholm, Sweden). A skin incision was performed and the skull was exposed and cleaned. Whole brain scan was performed using the PIM3 scanning camera to measure cerebral perfusion in both hemispheres (mainly the cortex) at baseline, after MCAO and at reperfusion. The change in mean perfusion of the ischemic hemisphere was expressed as a percentage of baseline.

Assessment of infarct volume and hemoglobin content

At 24 hours after the onset of MCAO, anesthesia was performed with Ketamine 44 mg/Kg and Xylazine 13 mg/Kg intra-muscular (cocktail), animals were then perfused with saline, sacrificed and their brains were removed. The brain tissue was sliced into seven 2 mm-thick slices in the coronal plane and stained with 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, Missouri, USA) for 15-20 minutes. Images of the stained sections were taken. Using image analysis software (Zeiss-KS300, Oberkochen, Germany), infarction zones were measured and infarct volume was calculated. The ischemic and non-ischemic hemispheres of the slices for the enzyme-linked immunosorbent assay (ELISA) were separated and processed, using the non-ischemic side as a control. After homogenizing the slices in the core of the infarct and taking the supernatants, ELISA was performed to measure the hemoglobin in the brain tissue(Hilali et al., 2004).

Neurological assessment

Neurological function was measured prior to reperfusion and at 24 hours (just before sacrifice) using the Bederson score(Bederson et al., 1986). Only animals with a score of 3 prior to reperfusion were included in all analyses. Before sacrifice, animals were tested again using the Bederson score. In a subset of controls and the high dose atorvastatin group, spontaneous activity of the rats in their cages was recorded continuously using telemetry. Data was recorded every 10 minutes for 48 hours before the stroke and until sacrifice at 24 hours after the onset of stroke. Percentage change in activity after reperfusion (after treatment) compared to baseline (before stroke) was calculated.

Molecular biomarkers:

Lipid peroxidation

Lipid peroxide concentration was determined by a method that measures the amount of thiobarbituric acid reactivity (TBARS) by the amount of malondialdehyde formed during acid hydrolysis of the lipid peroxide compound. 30 µl of sample was incubated with the reaction mixture containing 10 µl of 8.1% sodium dodecyl sulfate, 150 µl of 20% acetic acid solution (buffered to pH 3.5), and 150 µl of 0.8% thiobarbituric acid at 95°C for 1 hour. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1, v/v) were added and the final mixture was shaken vigorously. After centrifugation at 1500 x g for 10 minutes absorbance of the solvent layer was measured at 532 nm. Tetraethoxypropane was used to establish the standard curve and lipid peroxide level was expressed in terms of µmole/ L malondialdehyde per mg protein.

Detection of nitrotyrosine

Nitrotyrosine immunoreactivity is measured as an indicator of superoxide –dependent peroxynitrite formation by slot blot analysis. Brain homogenates (30 µg) prepared for immunoblotting experiments were immobilized onto a nitrocellulose membrane using a slot blot microfiltration unit. After blocking with 5% nonfat milk, membrane was incubated with an anti-nitrotyrosine antibody from Calbiochem and visualized with Pierce Super Signal Kit. The intensity of bands was analyzed by GelPro Software.

Western blot

Blots were performed for stroke and non-stroke sides of the brain homogenate. In brief, samples (50 µg protein of the brain homogenate) were separated on a 10% Sodium

dodecyl Sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk solution. Endothelial Nitric Oxide Synthase (eNOS), Phosphorylated eNOS (peNOS), serine/threonine protein kinase Akt (Akt), Phosphorylated Akt (pAkt) and actin proteins were determined by probing the membranes with the corresponding primary (cell signaling, Boston, MA) and secondary antibody (Santa Cruz, Santa Cruz, CA).

Blood Pressure:

In a subset of 24 rats (11 GK and 13 Wistar), telemetry transmitters (Data Sciences, Inc.) were implanted according to manufacturer's specifications under Na pentobarbital anesthesia (65 mg/kg, i.p; Abbott Laboratories). A midline incision was used to expose the abdominal aorta that was briefly occluded to allow insertion of the transmitter catheter into the abdominal aorta. The catheter was secured in place with tissue glue. The transmitter body was sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed using nonabsorbable suture (3-0). Rats were allowed to recover from surgery for 10 days and returned to individual housing for data acquisition before beginning stroke protocol. The individual rat cages were placed on top of the telemetry receivers and arterial pressure waveforms were continuously recorded throughout the study. Data was recorded every 10 minutes for 48 hours before the stroke and until sacrifice at 24 hours after the onset of stroke. Use of inhaled anesthetics during stroke surgery, with rapid recovery of consciousness, allowed frequent collection of arterial pressure data in awake animals close to the time of onset of ischemia.

Measurement of plasma atorvastatin levels:

The concentration of atorvastatin in plasma was determined by liquid chromatography tandem mass spectrometry (LC/MS/MS) as described previously (Jemal et al., 1999). The lower limit of quantification for atorvastatin was 0.2 ng/ml. The intra- and inter-day coefficients of variation were below 10% in the concentration range of 0.2 ng/ml to 200 ng/ml for plasma samples. Atorvastatin maximum concentration (Cmax) experiment was done on a subset of Wistar animals (n=3) while trough levels were measured in both treated Wistar and GK rats.

Statistical analysis:

The distributions of all continuous variables were checked for normality prior to analysis and a log transformation was found to be beneficial for infarct volume and excess Hgb. Baseline blood glucose (BG), weight, activity and MAP were compared for Strain and Drug differences using a 2X2 ANOVA without interaction. Bederson scores were compared using a 2 Strain (Wistar vs. GK) X 2 Drug (vehicle vs. atorvastatin) ANOVA where the interaction between Strain and Drug was investigated. Log infarct volume and log excess Hgb values were analyzed using a 2 Strain (Wistar vs. GK) X 3 Drug (vehicle vs. atorvastin5 vs. atorvastin15) ANOVA where the interaction between Strain and Drug was investigated. A linear contrast was used to the compare the 2 Strains across the levels of Drug and Dunnett's test was used for comparisons of each atorvastatin dose with vehicle. All molecular biomarkers were analyzed using a 2 Strain (Wistar vs. GK) X

2 Drug (vehicle vs. atorvastatin 15 mg/kg) X 2 Sides (stroke vs. non-stroke) repeated measures ANOVA. Changes from baseline for BG, weight, activity, and MAP were analyzed using a 2 Strain (Wistar vs. GK) X 2 Drug (vehicle vs. atorvastatin) analysis of covariance (ANCOVA) where baseline values were used as covariates prior to investigating the effects of Strain, Drug and their interaction. For blood pressure (BP) data, the average of all measurements prior to MCAO was the baseline value. Values obtained during the 3 hours of MCAO were averaged for the estimate of BP during stroke, the values for the period of the first 5 hours post-reperfusion were averaged for an estimate of the immediate effects of the drug, and all remaining values afterwards were averaged for the delayed post-reperfusion value. Means±SD are presented for the various groups. Vertical error bars in the graphs represent Standard error of the mean (SEM). SAS® version 9.1.3 was used for all analyses and $p<0.05$ was used to determine statistical significance.

Results:

At stroke, the weights among groups were similar. However, before sacrifice, control Wistar rats lost more weight than the diabetic GK rats (-46.5±12 vs. -35.5±7 gm, respectively; p=0.002) and change in weight was not affected by atorvastatin treatment. Baseline BG was significantly higher in the diabetic groups than in the normal rats groups (control and atorvastatin 15mg/Kg) (163.8±56 and 149.5±32 vs. 107±15 and 111.7±22 mg/dl, respectively; $p<0.0001$) (Table 1.). Hemoglobin A1c, which is a parameter to monitor chronic glycemic control in people with diabetes, was significantly higher in GK rats (n=9) than in Wistar rats (n=3) (6.7±0.34 vs. 4.2±0.05 % respectively; p=0.0018) indicating that the elevation in BG seen in the GK rats is due to chronic rather

than acute hyperglycemia. As expected, BG decreased in all groups when measured again at sacrifice (24 hours from stroke). After adjusting for baseline values, the decrease in serum glucose levels was found to be similar among the 4 different groups.

In a subset of untreated Wistar (n=8) and GK (n=9) rats, there was a similar 30% decline in cerebral perfusion in the right hemisphere (compared to the contralateral hemisphere) 3 hours after MCAO (33.3±6.2% in W vs. 33.6±9.9% in GK) (Figure 1). After reperfusion, blood perfusion in the ischemic hemisphere returned to the baseline level in both strains. Perfusion imaging was performed using PIM3 Doppler scanner.

Infarct volume, hemoglobin content and behavior:

As shown in Figures 2 and 3, atorvastatin-treated groups significantly lowered bleeding rate ($p=0.0011$) and infarct volume ($p=0.0007$) compared to their controls. The degree of reduction in bleeding rate ($p=0.0003$) and infarct volume ($p=0.0002$) in both strains differed by the dose of atorvastatin given indicating a positive dose response effect. By comparing the 2 doses of atorvastatin to the control groups, there was a significant reduction in hemoglobin content and infarct volume only in the higher dose group (15mg/Kg) ($p<0.05$). Infarct volume was reduced by about 10% with atorvastatin (5mg/Kg) and by more than 30% with atorvastatin (15mg/Kg) in the normal Wistar rats. Similarly, bleeding in the ischemic hemisphere was reduced by almost 40% with atorvastatin (5mg/Kg) and by 70% with the high dose atorvastatin (15mg/Kg) in Wistar rats. In GK rats, infarct volume and hemoglobin content were reduced by about 15% with atorvastatin (5mg/Kg) and 80% with atorvastatin (15mg/Kg). Since neurovascular

protection was achieved only with the higher dose of atorvastatin (15mg/Kg), all mechanistic studies included only this dose.

The neurovascular protection was associated with an improvement in the behavioral outcome in both diabetic and non-diabetic animals (Figure 4A). Atorvastatin (15mg/Kg) significantly improved Bederson score in both Wistar and GK rats (2.8±0.4 vs 2.5±0.4 points in Wistar and 3.0±0.1 vs. 2.8±0.3 points in GK rats (p=0.029). Hemorrhage formation in diabetic rats tended to be higher than in normoglycemic rats and they had significantly worse neurobehavioral outcome as shown by Bederson score (p=0.004), however their infarct volume was significantly smaller than normal Wistar rats (p<0.0001).

Using implanted transmitters, rats' spontaneous movement in the cages were recorded every 10 minutes throughout the experiment. By comparing rats' activity after treatments at reperfusion to their baseline activity levels before stroke, we found that there was a significant increase in the activity level after stroke in both Wistar and GK rats receiving vehicle compared to the atorvastatin-treated rats (70±23 vs -10±18% change from baseline, respectively, in Wistar and 37±32 vs -6±14% change from baseline, respectively, in GK rats, p=0.049 (Figure 4B). This is possibly due to an increase in the discomfort and loss of circadian rhythm in the control groups after stroke.

Molecular biomarkers:

Oxidative stress markers

To examine the differences between strains in oxidative stress levels and whether the beneficial effects of atorvastatin were associated with anti-oxidant effect, oxidative stress was assessed using a lipid peroxidation assay and nitrotyrosine slot blot. Both assays were done on both plasma and brain homogenate samples. Diabetes increased oxidative stress systemically as shown by plasma levels of lipid peroxides ($p<0.0001$) (Figure 5A) and nitrotyrosine ($p=0.001$) (Figure 5B) in control GK rats compared to Wistar rats. Interestingly, locally in the brain; diabetic animals had less oxidative stress than their normoglycemic controls as shown by brain lipid peroxides ($p=0.001$) (Figure 5C) and nitrotyrosine levels ($p=0.036$) (Figure 5D). Atorvastatin did not affect oxidative stress markers. As expected, stroke induced oxidative stress in both strains in the lipid peroxidation ($p=0.021$) and in the nitrotyrosine formation) ($p=0.04$).

Expression and phosphorylation of eNOS:

To examine the strain differences and whether the beneficial effects of atorvastatin were associated with a change in eNOS expression or activity, eNOS and peNOS protein amounts were quantified by immunoblotting in both the ischemic and the contralateral hemispheres of the brains. There were no differences between the diabetic and non-diabetic animals in eNOS expression or its phosphorylation. Stroke induced eNOS expression in both Wistar ($p=0.002$) and GK rats ($p=0.015$) but eNOS phosphorylation was less in the stroke side in Wistar ($p=0.007$) and GK rats ($p=0.011$). Atorvastatin did not affect eNOS expression but there was a trend towards an increase in eNOS phosphorylation in the ischemic hemisphere in GK rats treated with atorvastatin compared to control (69 ± 37 vs. 35 ± 23, p=ns). (Figure 6A and 6B)

Brain Akt Phosphorylation:

To further explore the strain differences and the mechanisms related to the neurovascular protection with atorvastatin, expression of pAkt was quantified similar to eNOS and peNOS. There was no difference between diabetic and non-diabetic rats. Atorvastatin (15mg/Kg) significantly increased Akt phosphorylation in the ischemic hemisphere in comparison with controls for both strains (p=0.006 for the interaction) (Figure 6C).

Blood pressure:

To explore the effect of diabetes, stroke, atorvastatin treatment and their interactions on blood pressure, we implanted the animals with BP transmitters and MAP was recorded every 10 min on telemetry prior to stroke, during the onset of ischemia, at reperfusion (a and then during the following 21 hrs until sacrifice next day. Baseline blood pressure was elevated in diabetes as shown in Figure 7 (MAP was 112±7 mmHg in GKs vs. 97±5 mmHg in Wistars, p<0.0001). In addition, onset of stroke in diabetes caused significantly higher elevation in blood pressure than in normoglycemic rats (34±5 mmHg in GKs vs. 26±6 mmHg in Wistars, p=0.02). Interestingly, atorvastatin (15mg/Kg) lowered the blood pressure in the diabetic rats for the first 5 post-reperfusion hours (141±9 mmHg in control GKs vs 133±9 mmHg in treated GKs) while there was no treatment effect on the MAP in the normoglycemic Wistar animals (p=0.023 for the interaction).

Atorvastatin concentrations after oral gavage:

Administration of atorvastatin (15mg/Kg) in Wistar rats (n=3) by oral gavage achieved an average (Cmax) (range) of 53.9ng/ml (27-77ng/ml) after 30 minutes. We also checked the trough levels (prior to sacrifice) of atorvastatin (15 mg/kg) in Wistar (n=8) and GK (N=5) rats and they were 7.5(1.4) ng/ml and 5.5(0.8) ng/ml respectively. Trough levels of atorvastatin (5mg/Kg) in Wistar (n=4) were proportionately lower at 2.5(0.7) ng/ml

Discussion:

Data from both animal and human studies suggest that hyperglycemia during acute ischemic stroke is associated with an increase in the brain injury due at least partially to an increase in blood brain barrier (BBB) permeability, edema and hemorrhage formation. This finally leads to worsening in functional outcomes and an increase in mortality (Bruno et al., 2004). We have adapted our stroke model to a model of type II diabetes mellitus (GK). We found that GK rats are more vulnerable to reperfusion injury and hemorrhage formation. This is due to the pathological remodeling characterizing this model which induces blood brain barrier breakdown leading to an increase in hemorrhage and worsening of the functional outcomes after stroke(Ergul et al., 2007). In GKs, Laser Doppler revealed a drop in cerebral perfusion after MCAo similar to that in Wistar rats. This experiment indicates that the small infarct size seen in the latter group is not due to inability to occlude the MCA. Bederson test showed that diabetic rats were more impaired when compared to Wistar rats regardless of the treatment, which may be due to the increased incidence of bleeding. Increased hemorrhage formation in

GK rats confirms that they are a suitable model to test vascular protective agents. As expected, diabetic rats had more oxidative stress than their normoglycemic controls systemically. However, in the brain, oxidative stress markers were lower in GK rats. These results also raise the question of whether the infarct, bleeding and oxidative stress pattern observed in GK rats is due to a compensatory response in this mild chronic diabetes model. As recently reviewed, our knowledge on the effect of diabetes of experimental ischemic stroke is limited to acute hyperglycemia or severe short term diabetes models, both of which showed increased infarct size and worse functional outcomes (Ergul et al., 2009). In future studies, we plan to study the effects of the duration and degree of hyperglycemia on the neurovascular and functional outcomes after stroke in healthy compared to diabetic animals.

The most important finding in our study is that, in both strains, atorvastatin (15mg/Kg) was not only neuroprotective but it was also vascular protective and improved neurological function. This result differs from that of the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial(Amarenco et al., 2006) in which atorvastatin was associated with an increase in hemorrhagic stroke, despite a robust reduction in overall stroke risk. This may be due to the difference between the acute versus the chronic effects of atorvastatin. In this project, we also found that the atorvastatin peak plasma concentration achieved after administering 15mg/Kg by oral route was similar to that reported after a dose of 80 mg/day of atorvastatin is given to humans(Lins et al., 2003). This gives our results clinical relevance for future translational studies, especially that 80 mg/day of atorvastatin was the dose shown to

reduce stroke recurrence in (SPARCL) trial(Amarenco et al., 2006). The failure of the lower dose atorvastatin to achieve statistically significant protection in our study, points to the importance of achieving high concentrations acutely in this population.

Another novel finding is that the vascular protection with atorvastatin (15mg/Kg) in the diabetic rats and their normoglycemic controls was associated with increased levels of phosphorylated Akt kinase in the brain homogenate, an effect that was shown by others to be involved in the acute neuro- and cardio-protection of statins (Wolfrum et al., 2004; Zhang et al., 2007; Prinz et al., 2008). The phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway is known to be very important in regulating cell growth, proliferation, and survival. The activation of the PI3K/Akt pathway reduces thrombogenicity, vascular permeability, inflammation and apoptosis and thereby preserves vascular function(Cantley, 2002). Statins induce Akt translocation to the plasma membrane of endothelial cells. Upon phosphorylation and subsequent activation of Akt at serine 473, it induces phosphorylation of Bad (member of the pro-apoptotic BCL2 protein family) , forkhead transcription factor(AFX), caspase 9, eNOS and inhibitor of nuclear factor kappa B (NFκB)(Datta et al., 1997). Although others have shown the importance of eNOS in statin neuroprotection(Endres, 2005), our study did not show any treatment effect on eNOS expression or its activation. We think that these differences are due to the prophylactic versus the acute effects of statins in stroke, the route of administration and the dosage administered. In parallel to our results, a previous study showed that atorvastatin in combination with tissue-type plasminogen activator reduced the disruption of vascular integrity in the brain after embolic stroke in rats without affecting

eNOS levels, and blocking NOS activity did not inhibit the beneficial effects of the combination treatment on stroke (Zhang et al., 2005). In addition, low doses of IV rosuvastatin after stroke provided neuroprotection and increased PAkt without altering eNOS expression or its phosphorylation(Prinz et al., 2008). However, in our study, it is also possible that the eNOS phosphorylation was transient with an early peak after atorvastatin administration followed by a decline to baseline level, a pattern that would not be detected using western blot on samples collected at 21 hours after reperfusion and drug administration.

Lastly, our study showed that these diabetic rats had slightly elevated blood pressure at baseline compared to their normoglycemic controls. This is in contrast to our earlier work, done in older GK animals, where there was no difference in blood pressure(Harris et al., 2008). It is likely that the endothelial response to the hyperglycemia changes over time and we are investigating this. The animals were also more sensitive to ischemia leading to a higher increase in their MAP than normal rats during MCA occlusion. These results are probably mediated by the endothelial dysfunction and the altered nature of the diabetic vasculature in GK rats(Harris et al., 2005; Elgebaly et al., 2008). Interestingly, atorvastatin (15mg/Kg) significantly reduced MAP after reperfusion for 5 hours, an effect that was only seen in GK rats. It is possible that this mild blood pressure lowering contributed to the robust protection seen in the diabetic animals in this study and may have improved their cerebral blood flow. Results from UCSD, a recent large, parallel-design randomized clinical trial, showed that various statins lower BP and this reduction may contribute to the cardiovascular protection of statins(Golomb

et al., 2008) . Although BP lowering is not clinically accepted as a tactic of neurovascular protection, we, as well as others, have already shown that various pharmacologic agents that lower BP are vascular protective after ischemic stroke(Hosomi et al., 2005) (Fagan et al., 2006; Elewa et al., 2007).

An interesting finding of our study is that we did not detect any effect of atorvastatin treatment on oxidative stress which may have been due to the following reasons. First of all, statins are known to reduce oxidative stress through their inhibitory effect on the Rac which inhibits NADPH oxidase and hence decrease ROS production(Endres, 2005). However, there are many other sources of oxidative stress and ROS in the brain after ischemia/reperfusion injury that are not inhibited by statins such as cycloxygenase, myeloperoxidase and the mitochondrial electron transport chain(Lo et al., 2003). In addition, ROS production seems to occur very early after ischemia which makes it a very difficult target to reach with the delayed oral statin treatment (given at 3 hours after the onset of stroke).

In conclusion, this is the first report of the robust vascular protective effect of acute, high dose atorvastatin in diabetic and normoglycemic rats. Atorvastatin is a novel vascular protective agent, already safely administered to many acute ischemic stroke patients, and may be especially helpful when given at reperfusion in high risk diabetic patients.

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Footnotes

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SCF has been a consultant or participated in a Speaker Bureau for Pfizer, Astra-Zeneca and Boehringer Ingelheim.

Legends for Figures

Figure 1

Change in cerebral perfusion 3 hours after MCAo measured with PIM2 scanner in W (n=8) and GKs (n=9). Perfusion was more than 30% lower in the ischemic hemisphere compared to the contralateral hemisphere (averaged over the entire hemisphere) and was not different among strains.

Figure 2

Effect of atorvastatin on the infarct volume in the ischemic hemisphere. Atorvastatin (15mg/Kg) significantly reduced infarct volume (mm^3) in both Wistars and GK rats (Third column white and black) compared to their controls (first column white and black) (* $p<0.05$). GK had significantly smaller infarct than Wistar rats (# $p<0.0001$). The degree of reduction in infarct volume was significantly related to dose in both strains ($p=0.0002$). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV 5: Atorvastatin 5mg/Kg; ATV 15: Atorvastatin 15mg/Kg

Figure 3

Effect of atorvastatin on the hemoglobin content in the ischemic hemisphere. Hemoglobin excess (µg Hgb/ g protein) decreased in atorvastatin(15 mg/Kg) groups (Third column white and black) compared to their controls (first column white and black) (* $p<0.05$). The degree of reduction in bleeding rate was significantly related to dose in both strains ($p=0.0003$). Vertical error bars indicate standard error of the mean (SEM).

W: Wistar; C: Control; ATV 5: Atorvastatin 5mg/Kg; ATV 15: Atorvastatin 15mg/Kg

Figure 4

A- Effect of atorvastatin 15 mg/Kg on Bederson score in the Wistar and GK rats subjected to MCAO. The animals were tested before reperfusion and treatment (white bars), and only animals with a score of 3 were further examined. Before sacrifice, Bederson score was significantly reduced with atorvastatin treatment in both strains (* p=0.0291). Bederson score was also significantly higher in GK compared to Wistar rats (# p=0.004). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV 15: Atorvastatin 15mg/Kg

B- Effect of atorvastatin 15 mg/Kg on activity level in Wistar and GK rats. Using implanted transmitters, rats' movement in the cages were recorded every 10 minutes throughout the experiment. There is a significant increase in the activity level in both Wistar and GK rats receiving methylcellulose compared to the atorvastatin-treated animals (* p=0.049). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV 15: Atorvastatin 15mg/Kg

Figure 5

Effect of strain and treatment on oxidative stress markers in both plasma (A &B) and brain (C & D). In plasma, both lipid peroxidation (A) and nitrotyrosine slot blot (B) showed significant elevation in the diabetic animals. In the brain, there was less oxidative stress in diabetic rats as shown in the lipid peroxidation assay (C). There were no differences between treated and control animals in plasma or the brain. W: Wistar;

C: Control; ATV 15: Atorvastatin 15mg/Kg; N:contralateral hemisphere; S: ipsilateral hemisphere.

Figure 6

Effect of atorvastatin and stroke on eNOS, PeNOS and PAkt levels in the brain.

(A) Quantitative data of percentage relative density showing increased eNOS expression in the ischemic hemisphere in Wistars (* p=0.002) and GK rats (# p=0.015). (B) eNOS phosphorylation was less in the stroke side in Wistar (* p=0.007) and GK rats (# p=0.011). (C) Quantitative data of percentage relative density showing increased activation of PAkt in atorvastatin-treated groups compared to their controls (* P=0.0007). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV 15: Atorvastatin 15mg/Kg; N:contralateral hemisphere; S: ipsilateral hemisphere.

Figure 7

Mean arterial blood pressure (MAP; mmHg) after acute stroke in the Wistar (triangles) and GK rats (rectangles). MAP was recorded every 10 min (telemetry) prior to stroke (baseline between 6 and 9 AM), during the onset of ischemia (at 10 AM; left arrow), reperfusion (at 1 PM; right arrow) and then during the following 21 hrs until sacrifice next day. At reperfusion, the animals were treated with methylcellulose (grey) or with atorvastatin (15mg/Kg) (black), another dose was given after 12 hours. The black horizontal bar indicates night-time in the light/dark cycles. Values shown are 1-h averages ± SEM. # p= 0.012, * p=0.023

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Table 1

	W control (n=7)	W ATV (n=8)	GK control (n= 7)	GK ATV (n=5)
Weight at stroke (SD)	290(8.5) g	286.1(6.9) g	286.3(8) g	289.2(9.5) g
Weight at sacrifice (SD)	243.5(14.3) g	237.4(11.1) g	250.8(9.1) g	251.9(14.8) g
BG at stroke (SD)	107(14.7) mg/dl	111.7(22.3) mg/dl	**163.8**(56.1) # mg/dl	149.5(31.9) # mg/dl
BG at sacrifice (SD)	101.2(22) mg/dl	109.5(41.2) mg/dl	121.3(38) mg/dl	130.4(44.5) mg/dl
Hemoglobin A1c (SD)	W Baseline (n=3)	N/A	GK Baseline (n=9)	N/A
	4.2(0.05)	N/A	6.7(0.34) *	N/A

Table 1

Weight, blood glucose (BG) and hemoglobin A1c (HA1c) levels among different treatment groups at baseline and at sacrifice. Wistar rats lost more weight than the diabetic GK rats (p=0.002). Baseline BG was significantly higher in GK than Wistar rats (# p<0.0001). HA1c was significantly higher in GK rats (* p=0.0018)

W: Wistar; C: Control; ATV: Atorvastatin 15mg/Kg

Figure 1

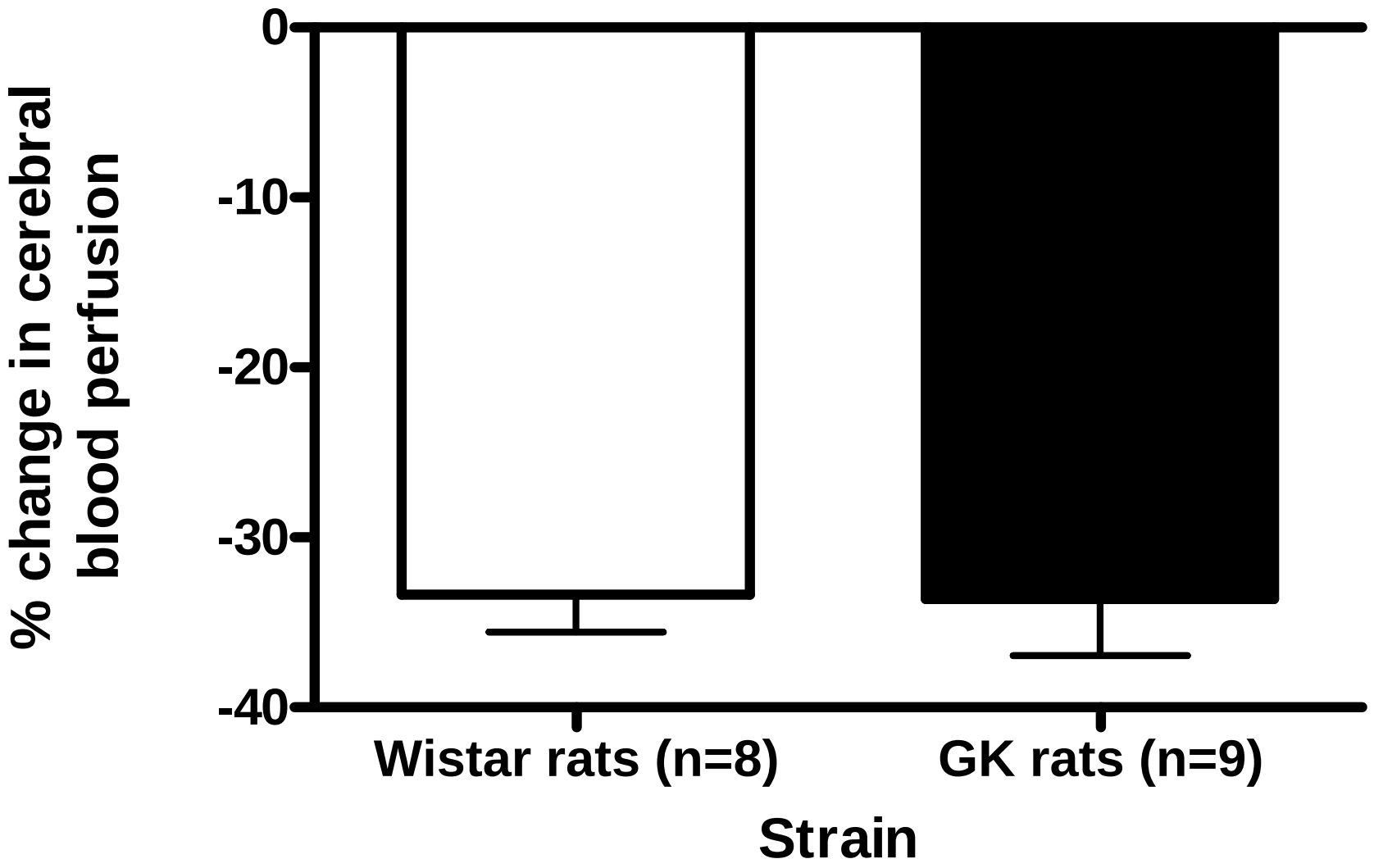


Figure 2

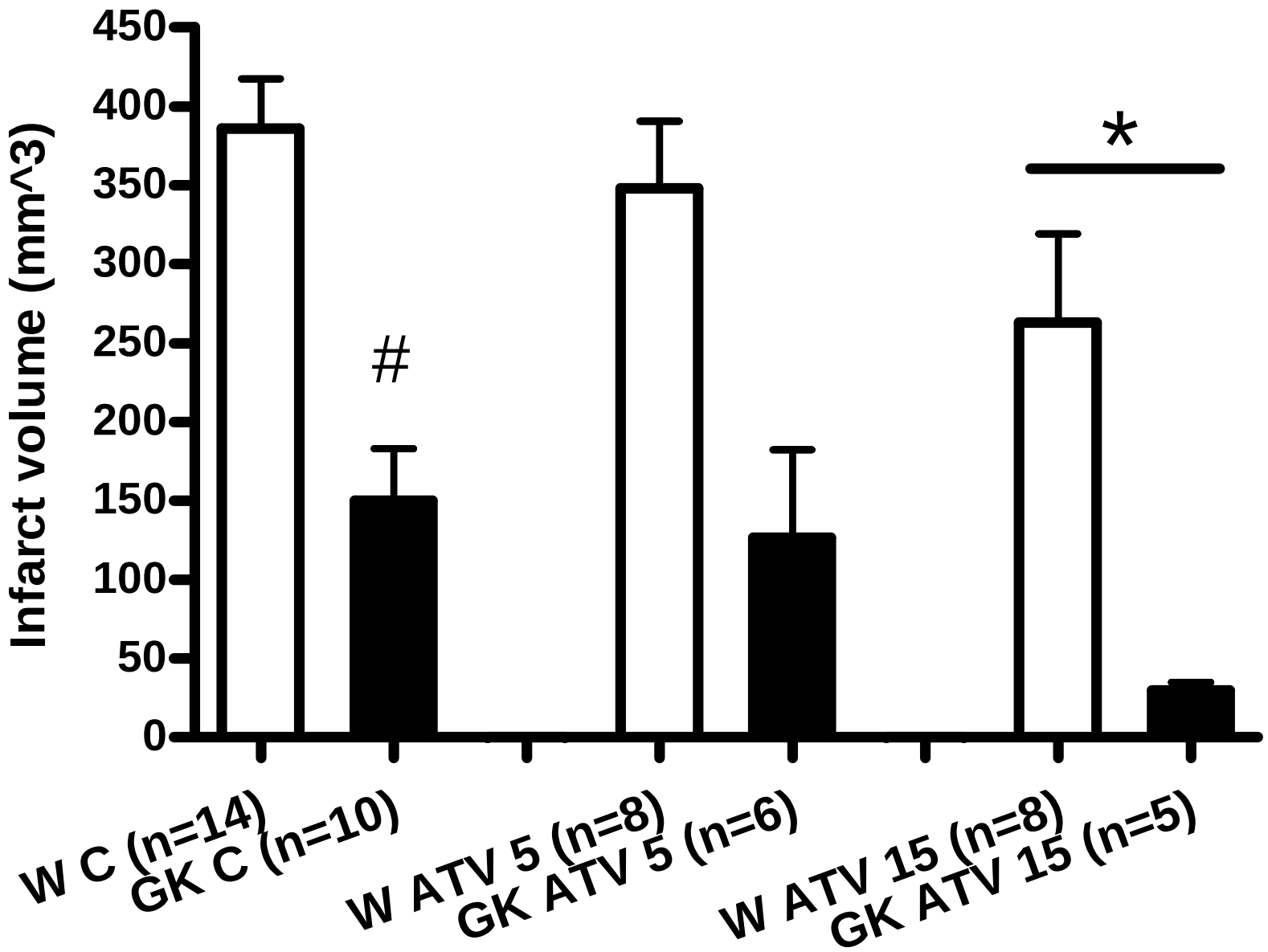


Figure 3

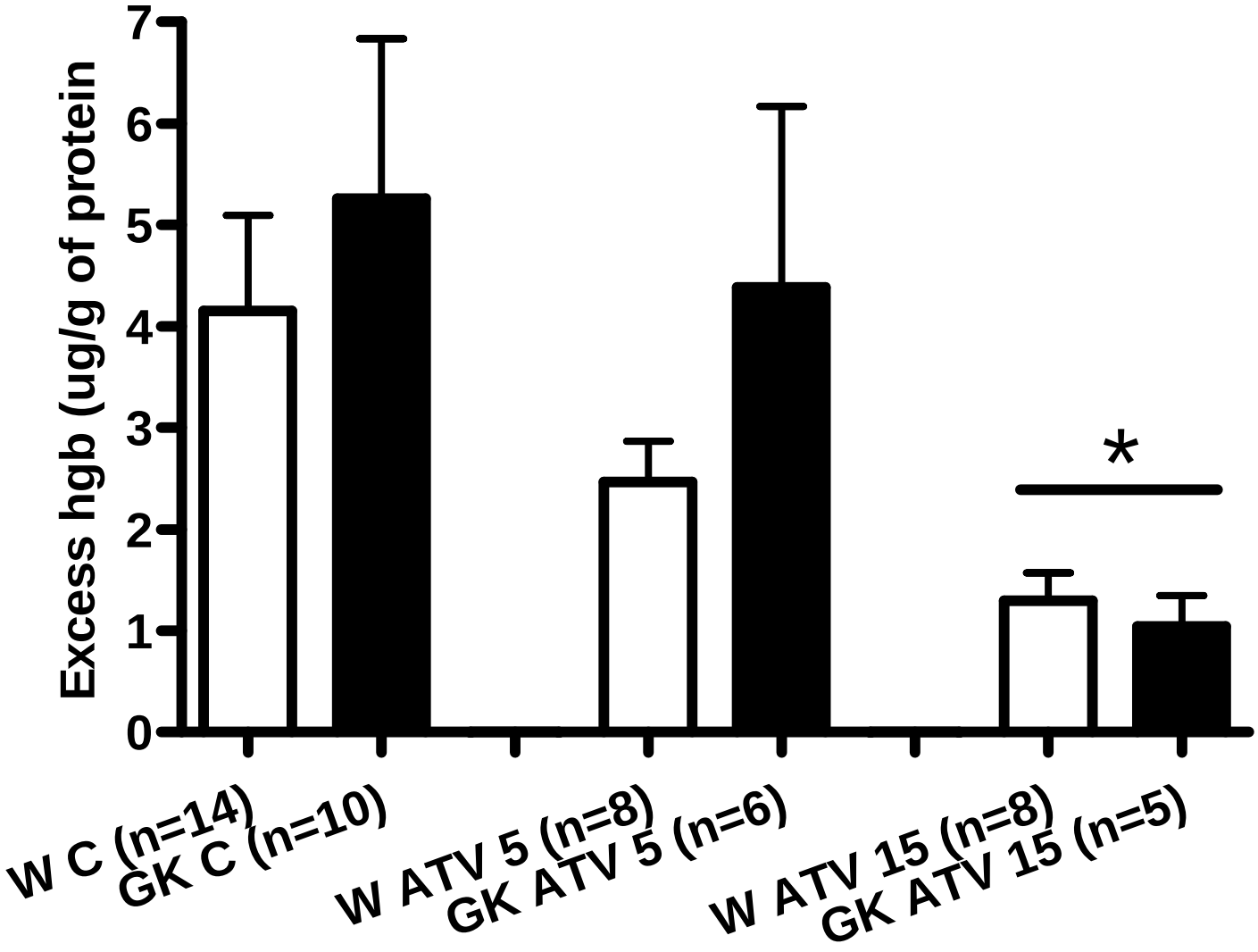
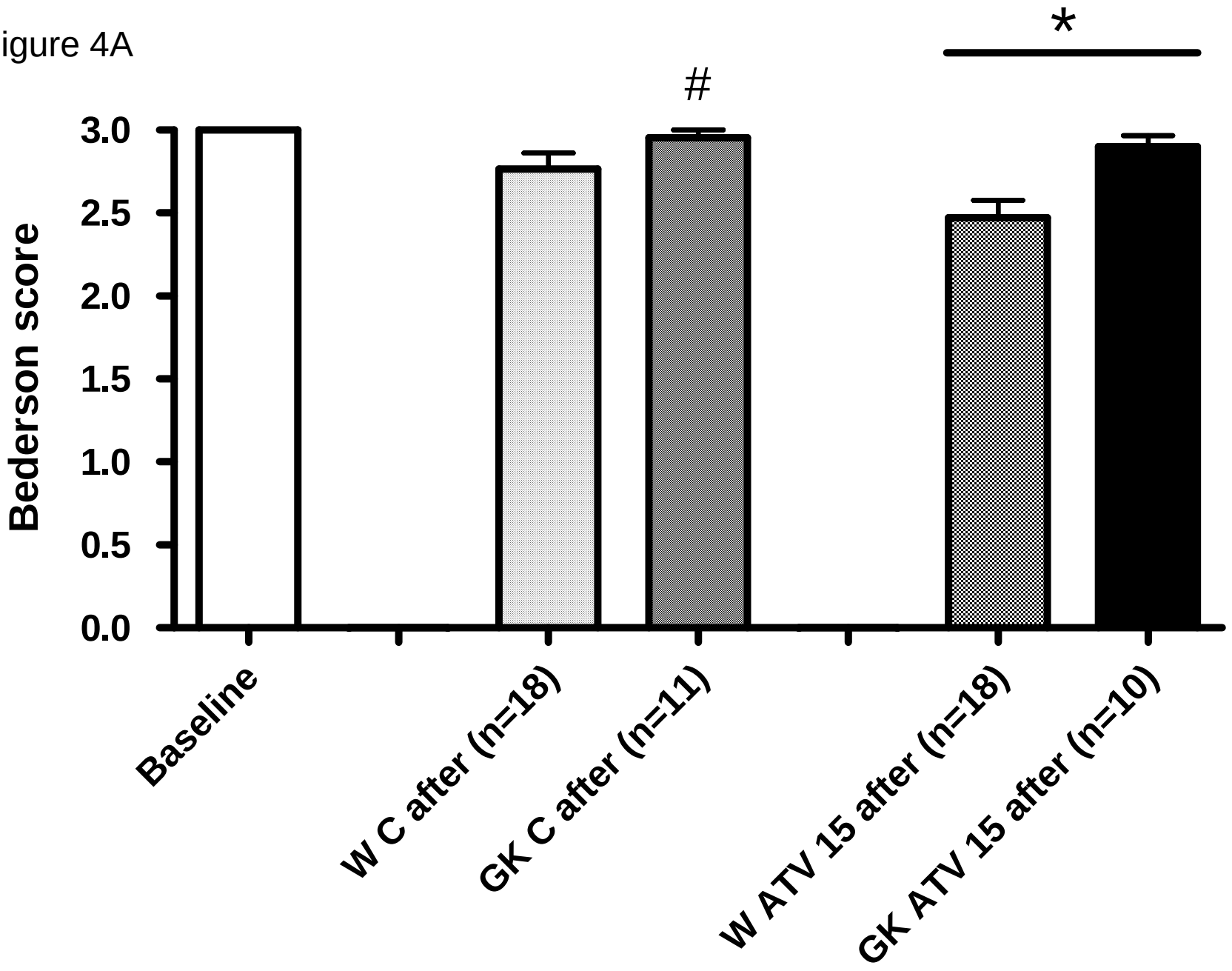


Figure 4A



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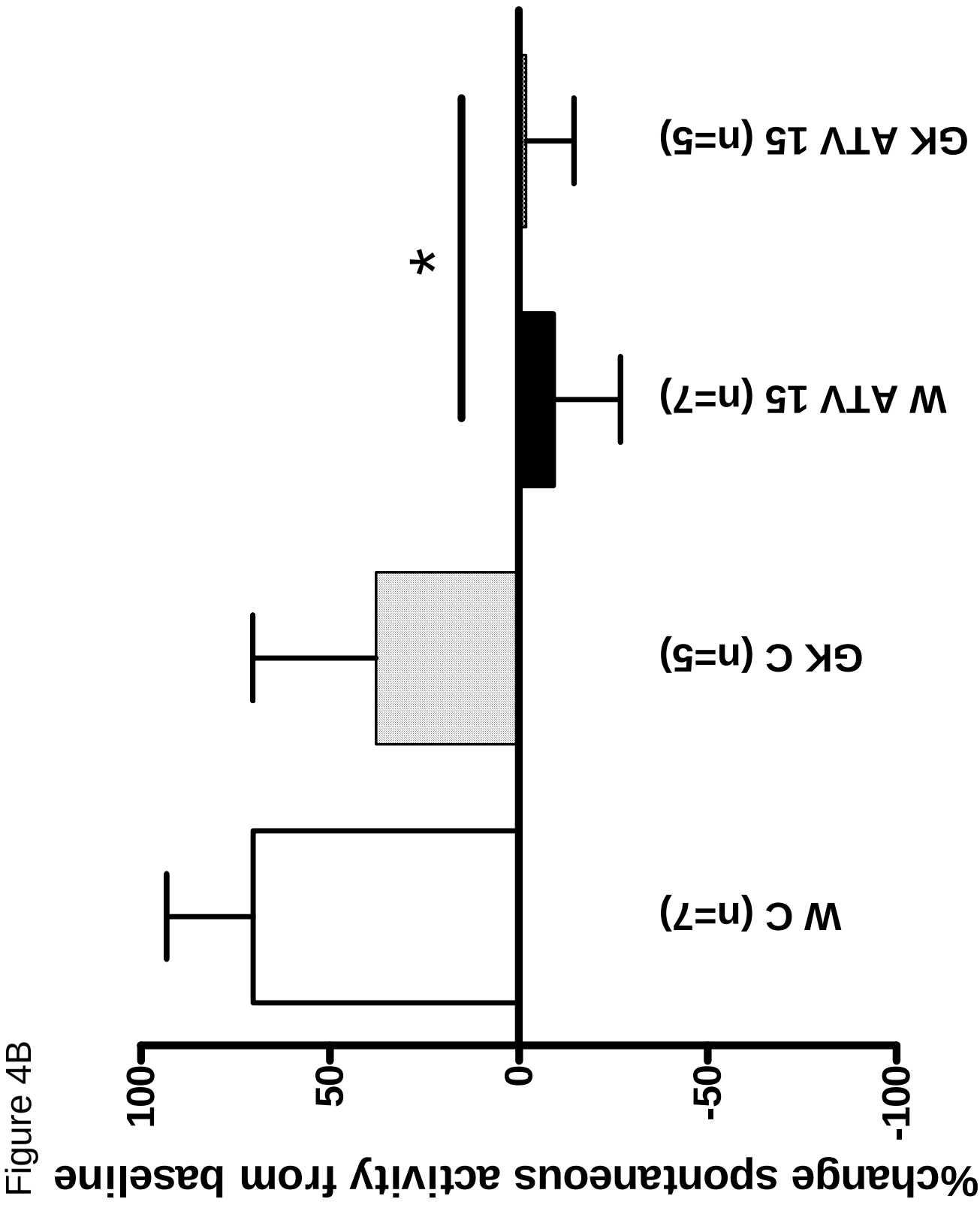
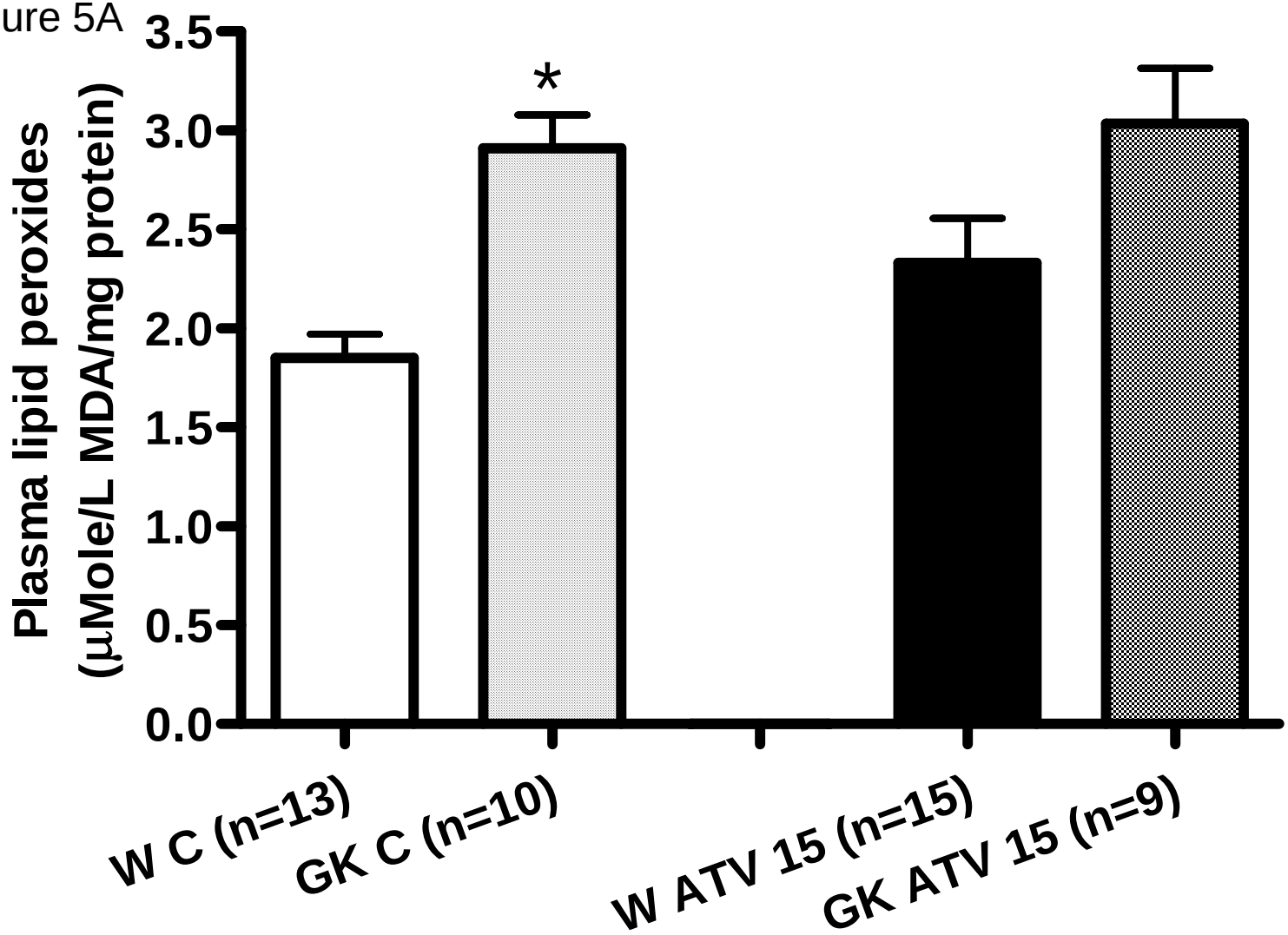


Figure 4B

Figure 5A



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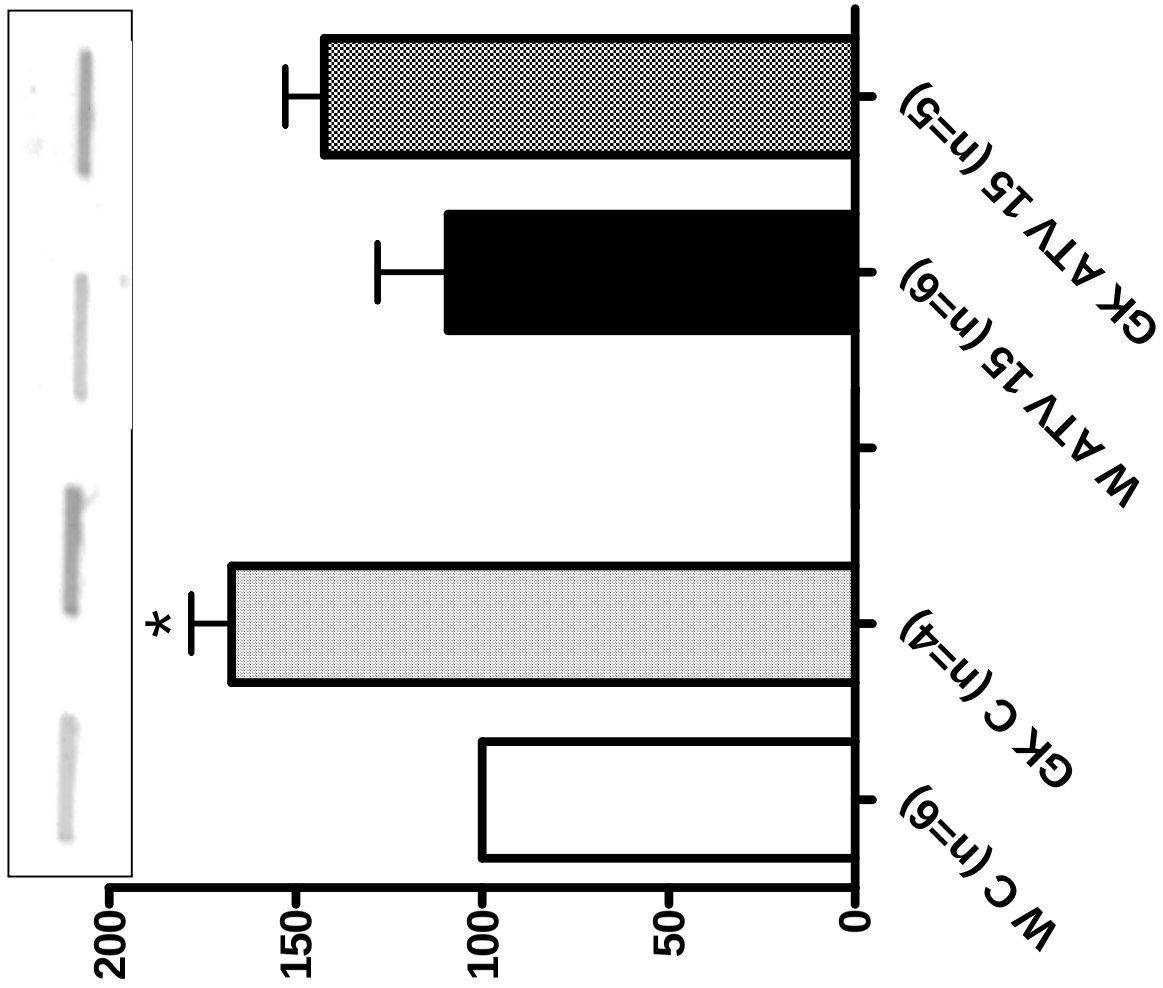

Figure 5B

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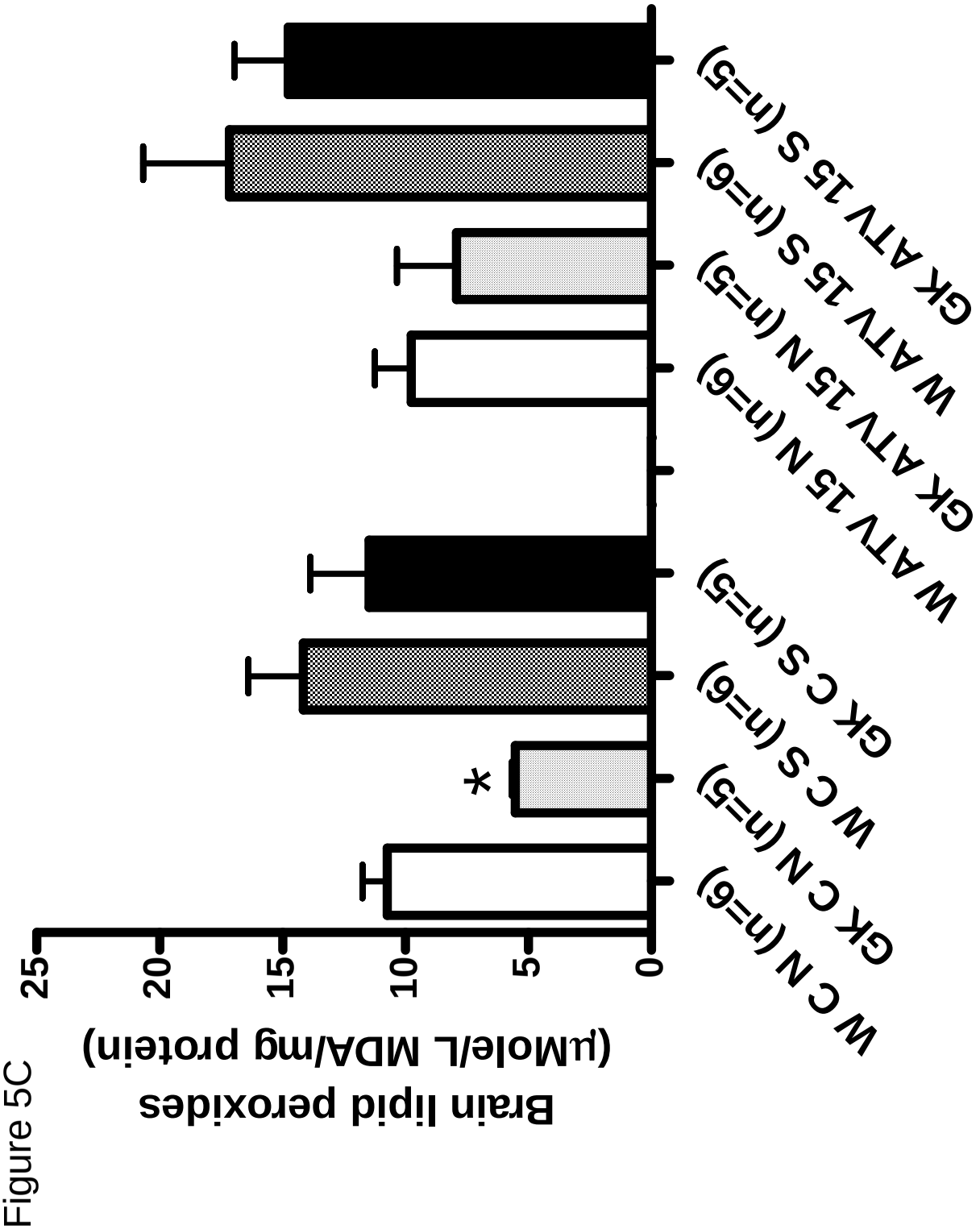
Figure 5C



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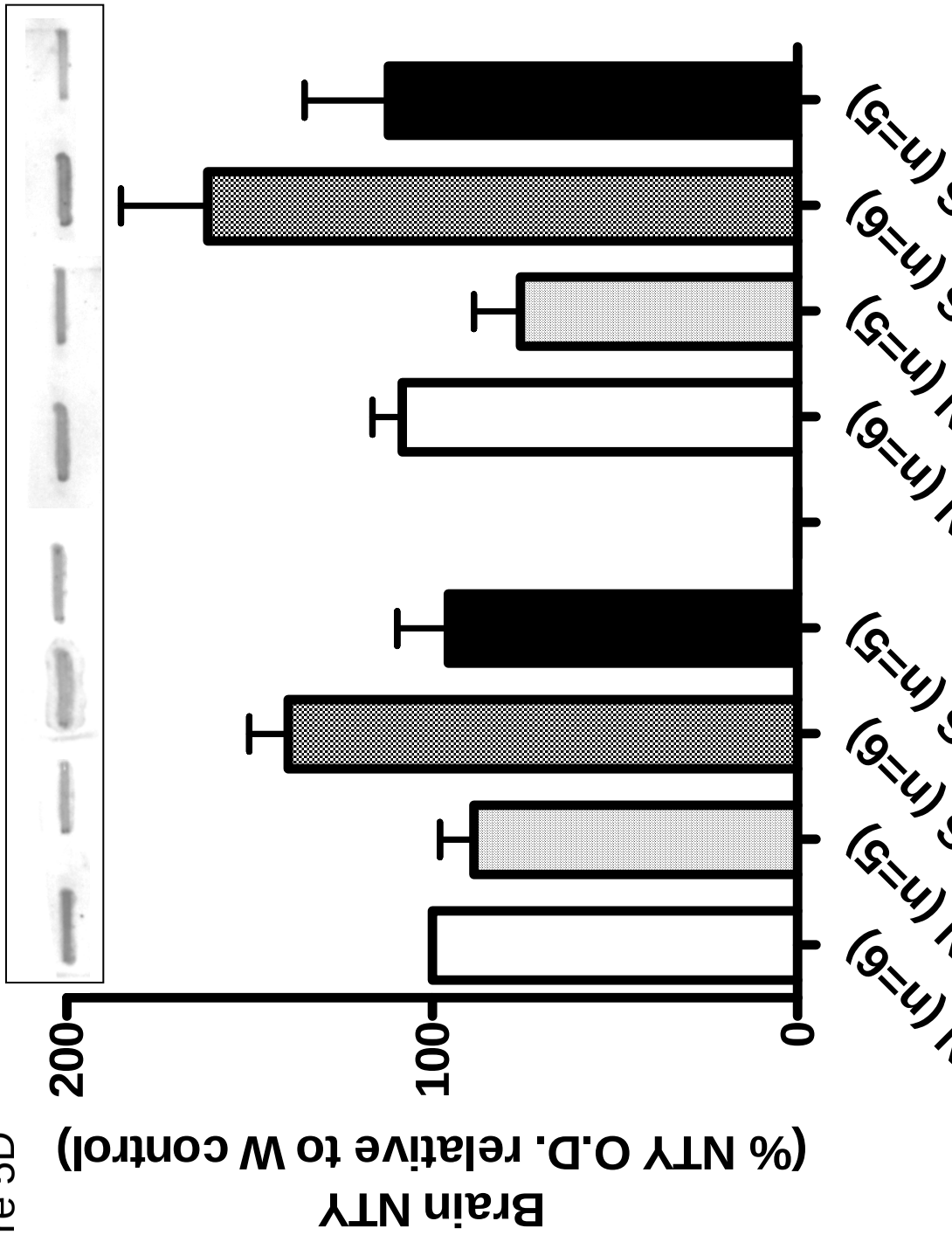
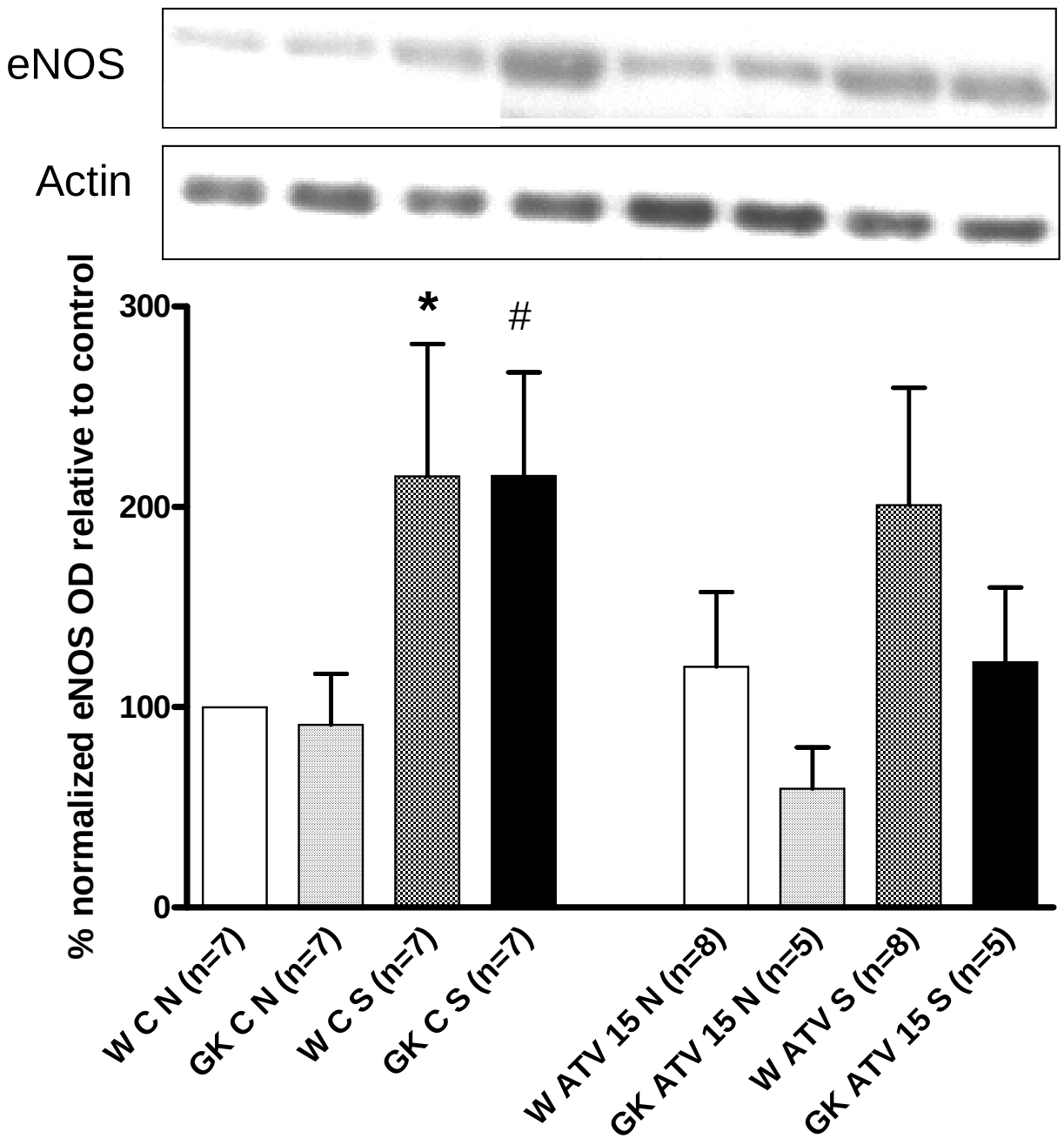


Figure 5D

Figure 6A



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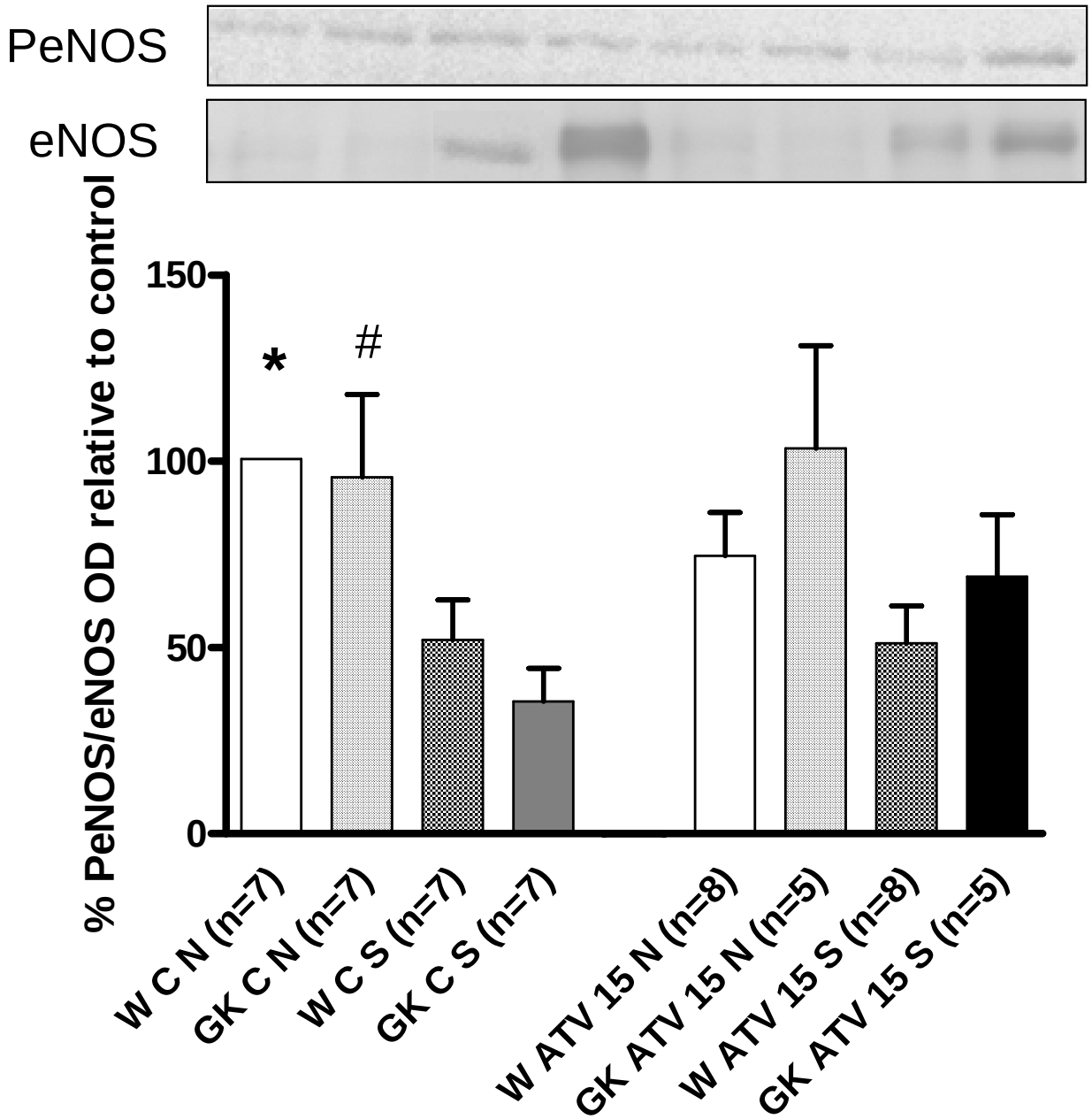

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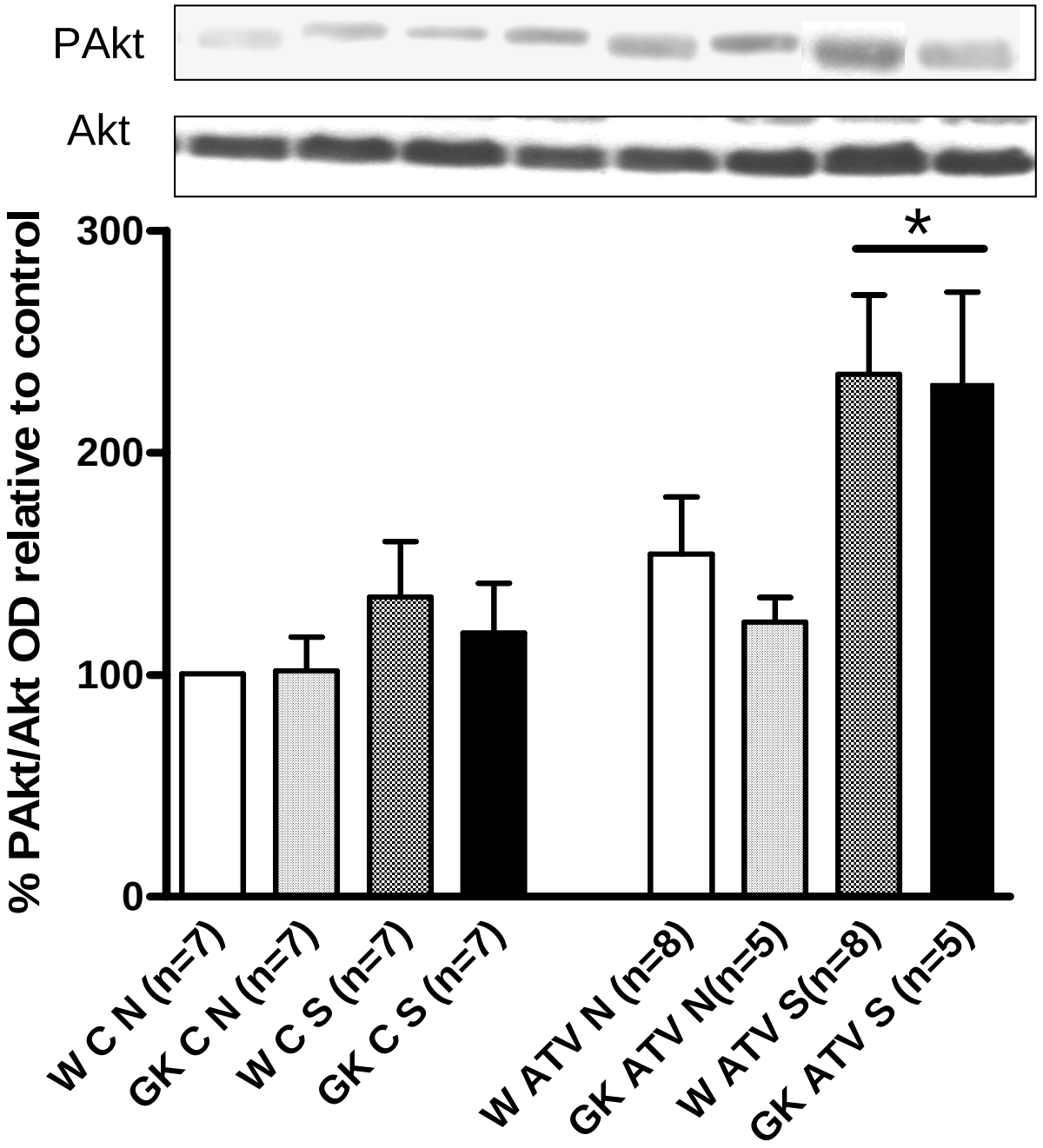

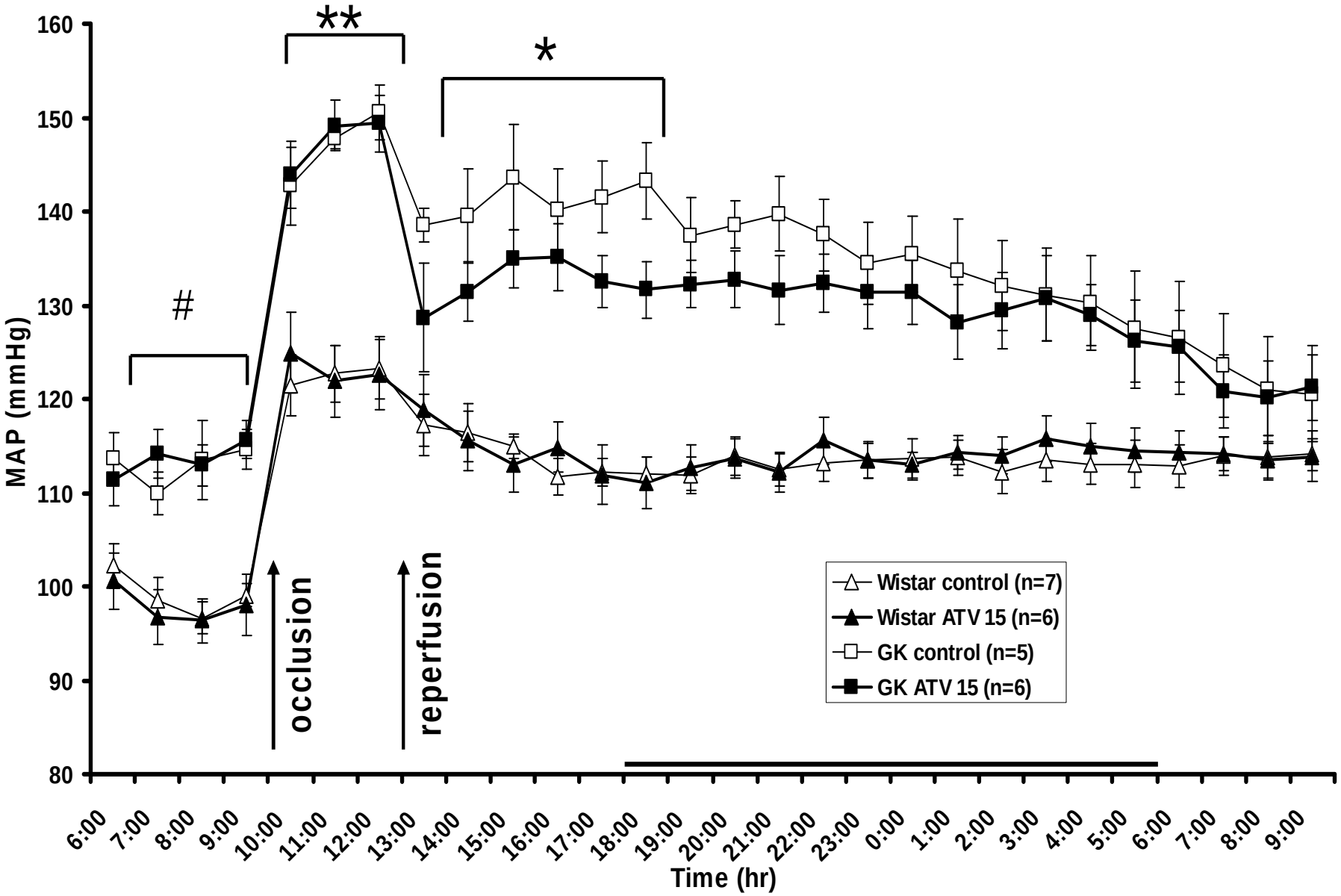

Figure 7