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José M González, Beatriz Somoza, M Victoria Conde, Maria S Fernández-Alfonso, M Carmen González, et al.. Hypertension increases middle cerebral artery resting tone in spontaneously hypertensive rats. Role of tonic vasoactive factor availability. *Clinical Science*, 2007, 114 (10), pp.651-659. <10.1042/CS20070361>. <hal-00479409>

HAL Id: hal-00479409

<https://hal.science/hal-00479409v1>

Submitted on 30 Apr 2010

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Hypertension increases middle cerebral artery resting tone in Spontaneously Hypertensive Rats. Role of tonic vasoactive factor availability

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Short title: Cerebrovascular resting tone in hypertension

Key words: resting tone, nitric oxide, middle cerebral artery, SHR, superoxide anion, confocal microscopy

Abstract

The present study explores the contribution of alterations in resting tone to cerebral artery narrowing in spontaneously hypertensive rats (SHR) and the role of hypertension development. Young pre-hypertensive and adult fully hypertensive SHR and their age-matched Wistar Kyoto rat controls were used. The contribution of basal vasoactive factors to resting tone was studied in middle cerebral arteries with pressure myography. Basal nitric oxide (NO) and superoxide anion ($O_2^{\cdot-}$) availability were determined with fluorescent indicators using confocal microscopy and lucigenin-enhanced chemiluminescence. Basal $O_2^{\cdot-}$ was also assessed in mesenteric resistance arteries. Middle cerebral arteries from adult, but not young pre-hypertensive rats, had augmented myogenic responses and resting tone and reduced relaxation to sodium nitroprusside compared to normotensive counterparts. Cerebral arteries from adult SHR also exhibited an increased tonic NO associated to a diminished basal $O_2^{\cdot-}$ availability. Basal $O_2^{\cdot-}$ was instead increased in SHR mesenteric arteries. The present results indicate that SHR large cerebral arteries increase their level of resting tone as a consequence of sustained hypertension and that this is related to a reduced NO responsiveness. We suggest that this increase in resting tone and myogenic responses could participate as a protective mechanism against the development of stroke in SHR. This study also demonstrates some unusual findings regarding current knowledge on NO/ $O_2^{\cdot-}$ balance in hypertension with important differences between vascular beds and draw attention to the complexity of this equation for cardiovascular health and disease.

Introduction

It is well known that large cerebral arteries are important contributors to cerebrovascular resistance and cerebral blood flow (CBF) regulation [1]. Brain vessels exhibit autoregulatory properties through several mechanisms. In addition to traditional considerations, the endothelium also participates in cerebral vascular autorregulation [2]. The endothelium, through the tonic release of vasoactive factors -mainly nitric oxide (NO)-, contributes to the resting tone of cerebral arteries and thus, participates in the control of cerebrovascular resistance and CBF [3, 4].

Increased peripheral vascular resistance to blood flow due to reduction of resistance artery lumen is a common finding in hypertension [5, 6]. This alteration is also present in brain vessels and cerebral artery narrowing has been demonstrated in several animal models of hypertension [7-12]. In addition to structural and mechanical abnormalities, functional changes might also participate in arterial narrowing [13]. However, despite the importance of resting tone for cerebrovascular resistance, its contribution to cerebral artery narrowing in hypertension and the role of tonic endothelial factor availability has not been thoroughly studied.

There is evidence that the above mentioned structural and mechanical alterations might be present in some vascular beds before hypertension development. Our own findings demonstrate that in mesenteric resistance arteries from spontaneously hypertensive rats (SHR), vessel stiffness is already present in 1-month old rats before significant increase in blood pressure [14]. Moreover, there is recent evidence that middle cerebral artery (MCA) narrowing due to a structural defect is also present in SHR at this age [12].

Based on this background, the first aim of the present study has been to assess, in pressurized MCA from SHR, the contribution of resting tone to internal diameter narrowing in young and adult rats. The second aim has been to assess the mechanism implicated in resting tone and to evaluate the contribution of tonic vasoactive factor availability, with particular attention to NO-superoxide anion ($O_2^{\cdot-}$) balance, which is known to be altered in hypertension [15-19].

Methods

Animals. Male 1-month old and 6-month old SHR and WKY rats were obtained from the colonies inbred at the Animal House of our Institution. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised in 1996) and with guidelines set by Spanish legislation (RD 1201/2005).

Pressure myography. MCA segments with endothelium were mounted on a pressure myograph (Model P100, J.P. Trading I/S, Aarhus, Denmark), as described [20]. Briefly, the artery was set to 10 mm Hg, allowed to equilibrate during 60 min at 37°C in gassed physiological salt solution in the absence of calcium (PSS; in mmol/l: 115 NaCl, 4.6 KCl, 25 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 EGTA, 11 glucose; pH 7.4). Thereafter, internal and external diameters (ID, ED) were measured at 20, 40, 60, 80, 100, 120 mm Hg. The segment was then set to PSS with calcium (2.5 mmol/l CaCl₂ omitting EGTA) and ID and ED were again measured at each level of pressure. From these data resting tone was calculated as follows: resting tone (%) = 100 × (ID_{0Ca} - ID)/ID_{0Ca}, where ID_{0Ca} and ID are internal diameters in 0Ca²⁺ and in PSS, respectively.

Vascular function was studied in segments pressurized at 70 mm Hg after a 60 min equilibration period in PSS. All experiments were performed in the presence of 0.1 µmol/l dexamethasone to avoid the contribution of induced nitric oxide synthase (iNOS). The involvement of tonic release of cyclooxygenase (COX) metabolites or NO to resting ID was determined comparing ID before and after addition of the specific inhibitors indomethacin (5 µmol/l), L-NAME (0.1 mmol/l) or S-methyl-L-thiocitrulline (SMTC, 10 µmol/l) to block COX, NOS and neuronal NOS (nNOS), respectively. Arterial contractility was determined by the response to 75mmol/l KCl as follows: KCl response (%) = 100 × (ID_{0Ca} - ID_{KCl})/ ID_{0Ca}, where ID_{0Ca} and ID_{KCl} are internal diameters in 0Ca²⁺ and in PSS after addition 75 mmol/l KCl, respectively. Thereafter, the arteries were washed with PSS and cGMP-dependent vasodilatation was studied by the increase in ID induced by cumulative addition of sodium nitroprusside (SNP, 0.01 to 10 µmol/l) in the presence of

0.1 mmol/l L-NAME (to exclude the contribution of endogenous NO). SNP response = $ID_{SNP} - ID_{L-NAME}$, where ID_{SNP} and ID_{L-NAME} are internal diameters in the presence of L-NAME, with or without SNP, respectively. All drugs were purchased from Sigma Chemical Co (UK).

Determination of basal NO and $O_2^{\cdot -}$ availability in adult rats. Basal NO availability was determined in MCA by the fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2 DA) as described [21]. Briefly, MCA segments were stabilized in PSS (30 min, 37°C) and stained with 10 μ mol/l DAF-2 DA (15, 30 or 45 min in the darkness, 37°C in a shaking bath). DAF-2 DA negative controls were incubated in 0.1 mmol/l L-NAME throughout the experimental period. Thereafter, the segments were washed 3 times for 1 min in PSS and fixed in 4% paraformaldehyde. The MCA segments were mounted intact on slides and they were visualized with a Leica TCS SP2 confocal microscope (Leica Microsystems, Germany) using the 488nm/515nm line. Serial images of the medial layer were captured at identical conditions of brightness, contrast and laser power for all experimental groups. Fluorescence intensity was quantified from confocal projections of the serial images with Metamorph image analysis software (Universal Imaging Corporation, UK).

Dihydroethidium (DHE, 3 μ mol/l) was used to determine basal $O_2^{\cdot -}$ availability in MCA and 3rd order mesenteric resistance arteries using the 488nm/590-620nm line of the microscope [21]. MCA segments were studied at 3 incubation times, as previously described for DAF-2 DA protocol. Mesenteric arteries were only studied at 30 minutes incubation. Negative controls were incubated throughout the experimental period with 15 U/ml of superoxide dismutase (SOD).

In MCA segments $O_2^{\cdot -}$ availability was also tested using the lucigenin-enhanced chemiluminescence assay as described [22]. Isolated MCA segments did not yield enough signal and all the arteries from the Willis Circle were used. They were dissected, cleaned from surrounding tissue and incubated in PSS (30 min, 37°C). Background relative luminiscence units (RLU) were measured with a luminometer (OPTOCOMP I, Bacterial Systems, GEM Biomedical Inc.) in tubes containing 5 μ mol/l lucigenin before addition of

the tissue. Thereafter, the arteries were transferred to the tubes and RLU emitted integrated over 30 s intervals for 20 min. Finally, RLU were measured for another 20 min after the addition of the $O_2^{\cdot -}$ scavenger tiron (10 mmol/l). Basal levels of $O_2^{\cdot -}$ were calculated as the value of tissue plus lucigenin-containing PSS minus background and divided by tissue weight.

Statistical analysis

Results are expressed as mean \pm SEM, and n denotes the number of animals used in each experiment. Statistical analysis was performed with GraphPad prism 4 software using paired or unpaired Student's *t* test or two-way ANOVA with repeated measures on pressure factor, where appropriate, followed by a Bonferroni post-hoc test. A value of $p < 0.05$ was considered significant.

Results

Changes in intrinsic tone and myogenic responses with age. In the presence of calcium, MCA from adult, but not from young SHR, had a significantly smaller ID when compared to age-matched WKY (Figure 1A). MCA from adult SHR maintained a constant diameter between 80-120 mm Hg, with a slope of the internal diameter-pressure curve near zero values ($\text{SHR}_{\text{slope}}=0.02\pm0.1$). However, there was no steady-state diameter in age-matched WKY ($\text{WKY}_{\text{slope}}=0.51\pm0.1$; $p<0.01$ when compared to SHR). In young rats there was no difference between strains in the slope of the ID-pressure curve ($\text{SHR}_{\text{slope}}=0.24\pm0.1$; $\text{WKY}_{\text{slope}}=0.16\pm0.1$; $p=0.87$).

MCA from adult SHR exhibited significantly larger resting tone compared to age-matched WKY rats. This difference was not observed in young rats (Figure 1B).

Factors involved in resting internal diameter. Indomethacin did not modify ID in SHR or WKY rats (Figure 2A). However, the unspecific NOS inhibitor L-NAME significantly reduced ID in both strains (Figure 2B). This reduction was smaller in SHR ($21.0\pm3\%$) when compared to WKY counterparts ($36.6\pm4\%$; $p<0.01$). As a result L-NAME abolished the differences in ID between strains. The specific nNOS blocker SMTC produced a negligible reduction of internal diameter, which was similar in both strains (SHR, $3.0\pm2.0\%$; WKY, $1.0\pm0.5\%$, $p=0.18$).

In young rats L-NAME reduced internal diameter to a similar extent in SHR ($38.8\pm6\%$) and in WKY ($30.5\pm4\%$; $p=0.25$) (Figure 2C).

Responses to vasoactive factors. There was no difference between strains in the contraction elicited by 75 mmol/l KCl in young or in adult rats (Figure 3A).

Responses to the exogenous NO-donor SNP were studied in the presence of 0.1 mmol/l L-NAME to avoid interference with endogenous NO production. In young rats, SNP concentration-response curves were similar between strains. However, in adult rats SNP responses were significantly reduced in SHR when compared to age-matched WKY (Figure 3B). Adult WKY exhibited significantly larger SNP responses than young WKY

counterparts. There was no difference in SNP response between old and young SHR (Figure 3B).

Basal NO availability in MCA from adult rats. The fluorescence emitted by DAF-2 DA was located in the smooth muscle cell (SMC) cytoplasm. Fluorescence intensity was markedly reduced by 0.1 mmol/l L-NAME pre-incubation (Figure 4A) and it was directly proportional to the incubation time (Figure 4B). DAF-2 DA fluorescence intensity was significantly larger in MCA from SHR when compared to age-matched WKY (Figure 4C).

Basal $O_2^{\cdot-}$ availability in MCA and mesenteric arteries from adult rats.

DHE fluorescence was located in the cell nuclei (Figure 5A and 6A), it was reduced by pre-incubation with the $O_2^{\cdot-}$ scavenger SOD (Figure 5A) and fluorescence intensity was directly proportional to the incubation time in both WKY and SHR MCAs (Figure 5B). In MCA DHE fluorescence intensity was significantly smaller in arteries from SHR when compared to age-matched WKY (Figure 5C). Lucigenin assay also showed significantly smaller RLU in cerebral arteries from SHR when compared to age-matched WKY (Figure 5D). This luminiscence was due to $O_2^{\cdot-}$ production as shown by its reduction by the $O_2^{\cdot-}$ scavenger tiron (data not shown).

In mesenteric arteries the opposite effect was found, with increased DHE-derived fluorescence in SHR arteries compared to WKY age-matched counterparts (Figure 6).

Discussion

The present work demonstrates the contribution of an increased resting tone to middle cerebral artery narrowing in adult SHR. The alteration in resting tone is not present in young pre-hypertensive SHR, and is therefore likely to be consequence of hypertension. These changes might contribute to the suggested protection against haemorrhagic stroke in this strain. This study also demonstrates reduced responsiveness of SMC to NO, but a larger tonic NO availability related to the unexpected finding of a reduced presence of $O_2^{\cdot-}$, which seem to be specific to the cerebral vasculature of SHR. To the best of our knowledge this is the first report assessing under non-stimulated conditions the role of vasoactive factor availability to resting cerebral artery tone in hypertension.

Internal diameter reduction due to structural alterations has been reported in brain vessels from several rat models of hypertension including SHR [7-12]. We have studied the possible contribution of resting tone to cerebral artery narrowing with pressure myography; the method which best resembles vascular physiological conditions *in vitro*. Our results show an increase in resting tone in adult SHR, similarly to previous findings in cerebral vessels from Brattleboro hypertensive rats [10]. In addition to an increase in resting tone, MCA from adult SHR also exhibited elevated myogenic responses, as previously described [23]. We aimed to determine if these alterations are consequence of high blood pressure or if they are present before hypertension development. We therefore, studied 1-month old SHR which, as shown by several authors are in the pre-hypertensive phase [12, 24, 25]. In the rat colonies inbred at our Institution we have previously demonstrated that at this age SHR exhibits similar blood pressure levels compared to age-matched WKY counterparts [14]. Resting tone and myogenic responses were similar in MCA from young SHR and WKY rats, suggesting that the described alterations observed in adult SHR are consequence of high blood pressure.

We analyzed the implication of tonic vasoactive factor release in the enhanced level of resting tone in adult SHR. The COX inhibitor indomethacine

did not modify internal diameter in SHR or WKY rats, suggesting no major contribution of COX-derived metabolites on MCA resting tone.

We also assessed the role of tonic NO availability to resting tone. In mammals, there are three NOS genes: neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2) and endothelial NOS (eNOS; NOS3) and unspecific and selective inhibitors have been developed to study the contribution of each of these enzymes to vascular tone regulation and vascular pathology. Regarding pharmacological agents, L-NAME and NG-monomethyl-L-arginine (L-NMMA) have been commonly used as unspecific NOS inhibitors [26-28]. With respect to the inducible form (iNOS), both the specific blocker aminoguanidine and dexamethasone –through inhibition of iNOS expression– have been used [26,28]. On the other hand, SMTC is a selective nNOS inhibitor which has been shown to be approximately 17-fold more selective for nNOS compared with eNOS [29]. We have previously demonstrated that iNOS can be induced in arteries during prolonged experiments [26]. Therefore, to study the contribution of NO to vascular tone in MCA we used dexamethasone throughout the experimental protocol to avoid interferences with iNOS. L-NAME reduced effectively MCA ID from both strains at the two age points studied, while the specific nNOS inhibitor SMTC had negligible effect. The dose of SMTC used in the present study (10 μ M) has been previously shown to elicit a pronounced and selective blockade of nNOS [30]. Since eNOS is the major NOS isoform expressed in the cardiovascular system and we avoided iNOS, we can conclude that L-NAME is inhibiting endothelial NO, which seems to be the main factor regulating resting tone in MCA. This is in accordance with previous studies showing that NO is key in the regulation of CBF and basal cerebrovascular tone [3, 4, 31].

In adult rats L-NAME had a smaller effect on SHR arteries compared to WKY counterparts and it abolished the differences in internal diameter between strains. Thus, increased resting tone in SHR seems to be related to an alteration in NO-pathway. The reduction of ID by L-NAME is the consequence of: 1) the responsiveness of SMC to NO and 2) the availability of NO to SMC, which in turn, depends on how much is produced and how much is destroyed by $O_2^{\cdot -}$. We, therefore, analyzed if the smaller effect of L-NAME on

SHR MCA was due an alteration in the responsiveness of the MCA to NO and/or to a defect in NO availability.

To study the responsiveness of SMC to NO we analyzed the relaxation elicited by an exogenous NO donor (SNP). In these experiments we blocked NOS with L-NAME to avoid interference with endogenous NO. Under these experimental conditions MCA from adult SHR showed a smaller relaxation to SNP when compared to WKY counterparts. However, responses to the unspecific contractile agent KCl were not different between strains. These results suggest that the cerebral vasculature of SHR has a reduced responsiveness to NO compared to the normotensive adult WKY; which is not related to a general impairment of SMC reactivity. This is in accordance with previous studies which demonstrate that local the CBF increase to infusion of the NO-donor SIN-1 is reduced in SHR compared to WKY rats [32]. The reduced response to NO observed in MCA has also been reported in other vascular beds from SHR and stroke-prone SHR and it has been linked to a decreased expression of soluble guanylyl cyclase [33-35]. The analysis of the responses to SNP in relation to age demonstrated that in MCA from 1 month-old rats there are no differences between strains. Thereafter, MCA from WKY increases sensitivity to the NO-donor with age, while SHR MCA does not. Similar results were found in SHR aorta, where YC-1-induced content of cGMP in aortic rings was similar in young SHR and WKY rats and it increased with age in WKY, but not in SHR [35].

We also analyzed the second possibility, i.e. that a reduced NO availability could also contribute to the reduction in L-NAME responses. To determine the NO available to SMC, we used the fluorescent indicator DAF-2 DA. This compound is captured by vascular cells and in the presence of NO and oxygen produces a highly fluorescent compound in the cell cytoplasm, which is proportional to the amount of NO [36,37]. We have previously used confocal microscopy and image analysis software to quantify NO availability from several vascular beds [21,38]. This method was sufficiently sensitive to enable quantification of NO tonic availability in MCA segments, as demonstrated by the cumulative increase in fluorescence which was blocked by L-NAME. Fluorescence was significantly larger in SHR MCA compared to WKY counterparts, indicating that, under basal conditions, SHR cerebral

arteries exhibit a larger NO availability. The amount of NO available depends on the balance between NO production and inactivation, mainly due to $O_2^{\cdot-}$ [17, 39]. In order to determine if the observed differences between strains in tonic NO was related to an alteration in $O_2^{\cdot-}$ we used DHE, a sensitive method which enables to detect $O_2^{\cdot-}$ in single arterial segments [21]. Our data showed a decreased basal $O_2^{\cdot-}$ in SHR MCA. This was confirmed by the lucigenin-enhanced chemiluminescence. This method is less sensitive than DHE and a larger amount of tissue (all the arteries from the Willis Circle) was required to detect luminescence under basal conditions. This reduction of $O_2^{\cdot-}$ explains the higher NO availability in SHR MCA and might be surprising, given the fact that increased oxidative stress has been thoroughly reported in hypertension [15-19]. The reduced presence of $O_2^{\cdot-}$ in SHR seems to be specific to the cerebral vasculature, as in 3rd order mesenteric arteries from age matched rats, basal $O_2^{\cdot-}$ was instead increased in SHR. These data are in accordance with several other studies performed in both conduit [40,41] and resistance arteries [42] from this strain.

The decreased basal $O_2^{\cdot-}$ in SHR cerebral vessels could be explained by a higher SOD activity in the hypertensive strain, able to quickly scavenge tonic $O_2^{\cdot-}$ production. In this regard, Paravicini and co-workers demonstrated that it was not possible to detect basal production of $O_2^{\cdot-}$ with chemiluminescence in single basilar artery segments from SHR and WKY rats. They also demonstrated that, when SOD activity was blocked, basal $O_2^{\cdot-}$ production could be detected, but only in SHR [43]. It has also been demonstrated that application of NADPH to basilar arteries caused larger vasodilatation in SHR than WKY [44]. It has to be taken into account that $O_2^{\cdot-}$ dismutated by SOD produces H_2O_2 which is a powerful dilator in cerebral vessels [43, 45-47]. Since NADPH oxidase activity is higher in SHR cerebral vessels [43], the increased vasodilatation to NADPH in SHR [44] is likely to be associated with a parallel increase of SOD activity in the hypertensive strain.

Regarding the consequences of the present findings for cerebrovascular regulation, we suggest that the functional changes observed

in adult SHR could act as a protective mechanism against the development of haemorrhagic stroke. In accordance with this hypothesis is the demonstration that the autorregulatory properties of the MCA are impaired in stroke-prone SHR, when compared with the stroke resistant SHR [11]. This has also been observed in uremic hypertension [48] and it has been proposed that an alteration of autorregulatory properties might be a key step in stroke development [11, 49]. We, therefore, suggest that the observed changes in resting tone and myogenic responses are a functional adaptation that takes place along with high blood pressure rise in SHR. Regarding the influence of oxidative stress/NO balance to regulation of cerebrovascular tone, the conclusion is less evident. In the cerebral circulation $O_2^{\cdot -}$ exerts a dual role on vasodilator balance, contributing to NO destruction on one hand, and as source of H_2O_2 generation, a powerful vasodilator for brain vessels, on the other [50]. In this context, the term 'oxidative stress', and its association with cardiovascular pathology, may be an over-simplification that hides the complexity and diversity of reactive oxygen species. Therefore, further studies are needed to understand the role of "oxidative stress" and its implications for cardiovascular health in general and, in particular for cerebrovascular disease.

Acknowledgments. This work was funded by Ministerio de Educación y Ciencia, Spain (BFI 2001-0638 and BFU2004-04148). We are grateful to Carmen Fernández Criado in charge of the Animal House and Diego Megías for his technical assistance with the confocal microscope.

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Figure legends

Figure 1. (A) Internal diameter-pressure curves in the presence of calcium in middle cerebral artery (MCA) segments from 1-month (1m) and 6-month (6m) old SHR and WKY rats. (B) Effect of age on resting tone in MCA segments from SHR and WKY rats. Data are expressed as mean \pm SEM. In parenthesis it is shown the number of animals. Statistical analysis was performed by two-way ANOVA or unpaired Student's *t* test, where appropriate; * $p < 0.05$ SHR compared with age-matched WKY; # $p < 0.05$, 6-month old compared with 1-month old rats of the same strain.

Figure 2. (A) Effect of 5 $\mu\text{mol/l}$ indomethacin or (B) 0.1 mmol/l L-NAME on internal diameter of middle cerebral arteries (MCA) pressurized at 70 mm Hg from 6-month old (6m) SHR and WKY rats. (C) Effect of 0.1 mmol/l L-NAME on internal diameter of MCA from 1-month old (1m) SHR and WKY rats. Data are expressed as mean \pm SEM. In parenthesis is shown the number of animals. Statistical analysis was performed by paired or unpaired Student's *t* test, where appropriate. * $p < 0.05$ when compared with WKY; + $p < 0.05$, when compared with control.

Figure 3. (A) Responses to 75 mmol/l KCl in middle cerebral artery (MCA) segments from 1-month and 6-month old SHR and WKY rats. (B) Concentration-responses curves to sodium nitroprusside (SNP) in the presence of 0.1 mmol/l L-NAME in MCA pressurized segments from 1-month and 6-month old SHR and WKY rats. Data are expressed as mean \pm SEM. In parenthesis is shown the number of animals. Statistical analysis was performed by two-way ANOVA or Student's *t* test, where appropriate. * $p < 0.05$ SHR compared with age-matched WKY rats; # 6 month old WKY compared to 1-month old counterparts.

Figure 4. (A) Confocal projections of the medial layer of middle cerebral arteries (MCA) from 6-month old SHR and WKY rats stained with the NO-

fluorescent indicator DAF-2 DA and effect of 0.1 mmol/l L-NAME pre-incubation on SHR MCA. (B) Effect of DAF-2 DA incubation time on emitted fluorescence in MCA from SHR and WKY rats. (C) Quantitative data of DAF-2 DA fluorescence intensity with time in MCA segments from 6-month old SHR and WKY rats. Data are expressed as mean \pm SEM. In parenthesis is shown the number of animals. Statistical analysis was performed by unpaired Student's *t* test. **p*<0.05 when compared with WKY rats.

Figure 5. (A) Confocal projections of the medial layer of middle cerebral arteries (MCA) from 6-month old SHR and WKY rats stained with the $O_2^{\cdot-}$ fluorescent indicator DHE and effect of 15 u/ml SOD pre-incubation on SHR MCA. (B) Effect of DHE incubation time on emitted fluorescence in MCA from SHR and WKY rats. (C) Quantitative data of DHE fluorescence intensity with time in MCA from SHR and WKY rats. (D) Lucigenin-enhanced luminiscence in cerebral arteries from 6-month old SHR and WKY rats. RLU, relative luminiscence units. Data are expressed as mean \pm SEM. In parenthesis is shown the number of animals. Statistical analysis was performed by unpaired Student's *t* test. **p*<0.05 when compared with WKY.

Figure 6. (A) Confocal projections of the medial layer of mesenteric resistance arteries (MRA) from 6-month old SHR and WKY rats stained during 30 min with the $O_2^{\cdot-}$ fluorescent indicator DHE. (B) Quantification of DHE emitted fluorescence in MRA from SHR and WKY rats. Data are expressed as mean \pm SEM. In parenthesis is shown the number of animals. Statistical analysis was performed by unpaired Student's *t* test. **p*<0.05 when compared with WKY.

Figure 1

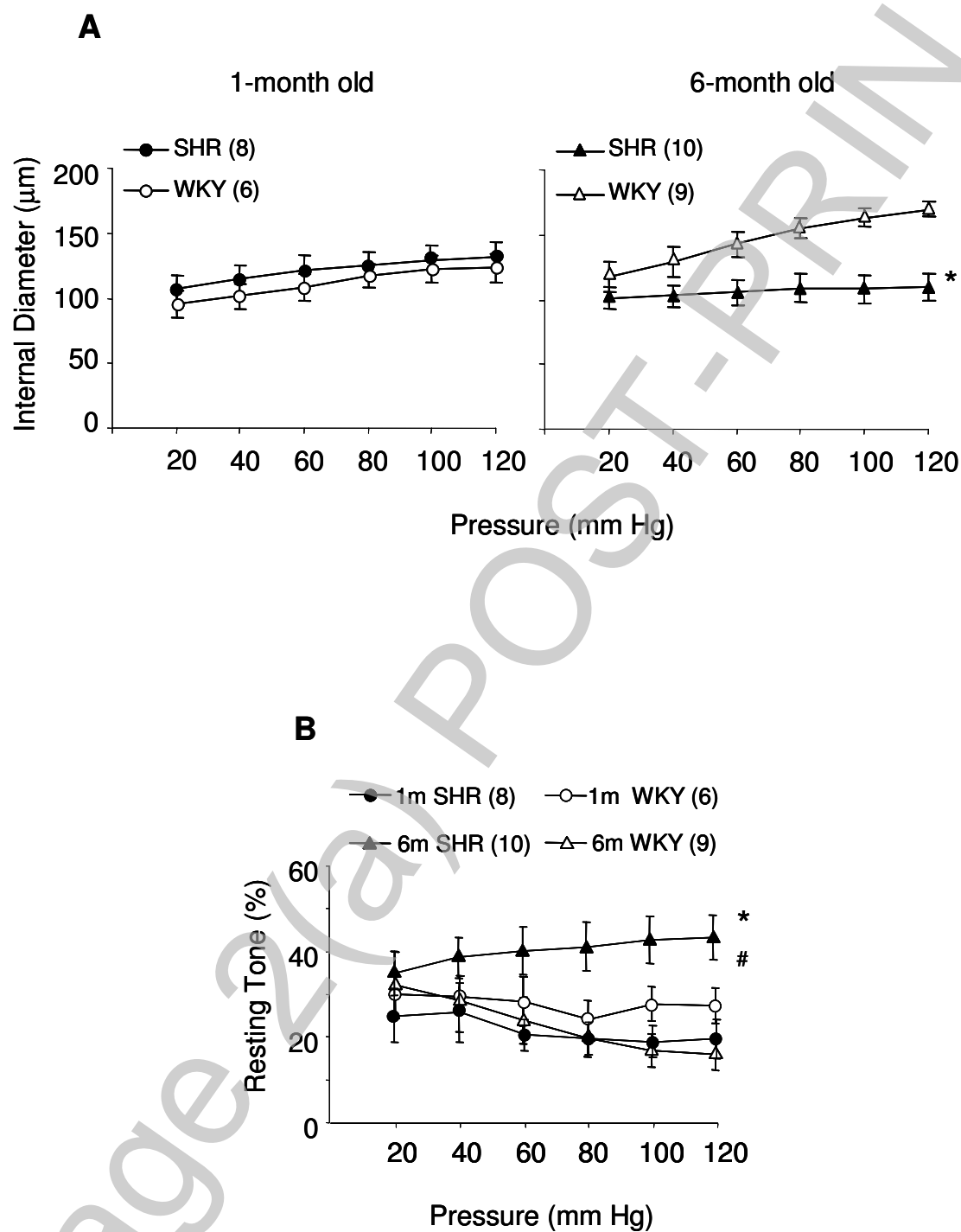


Figure 2

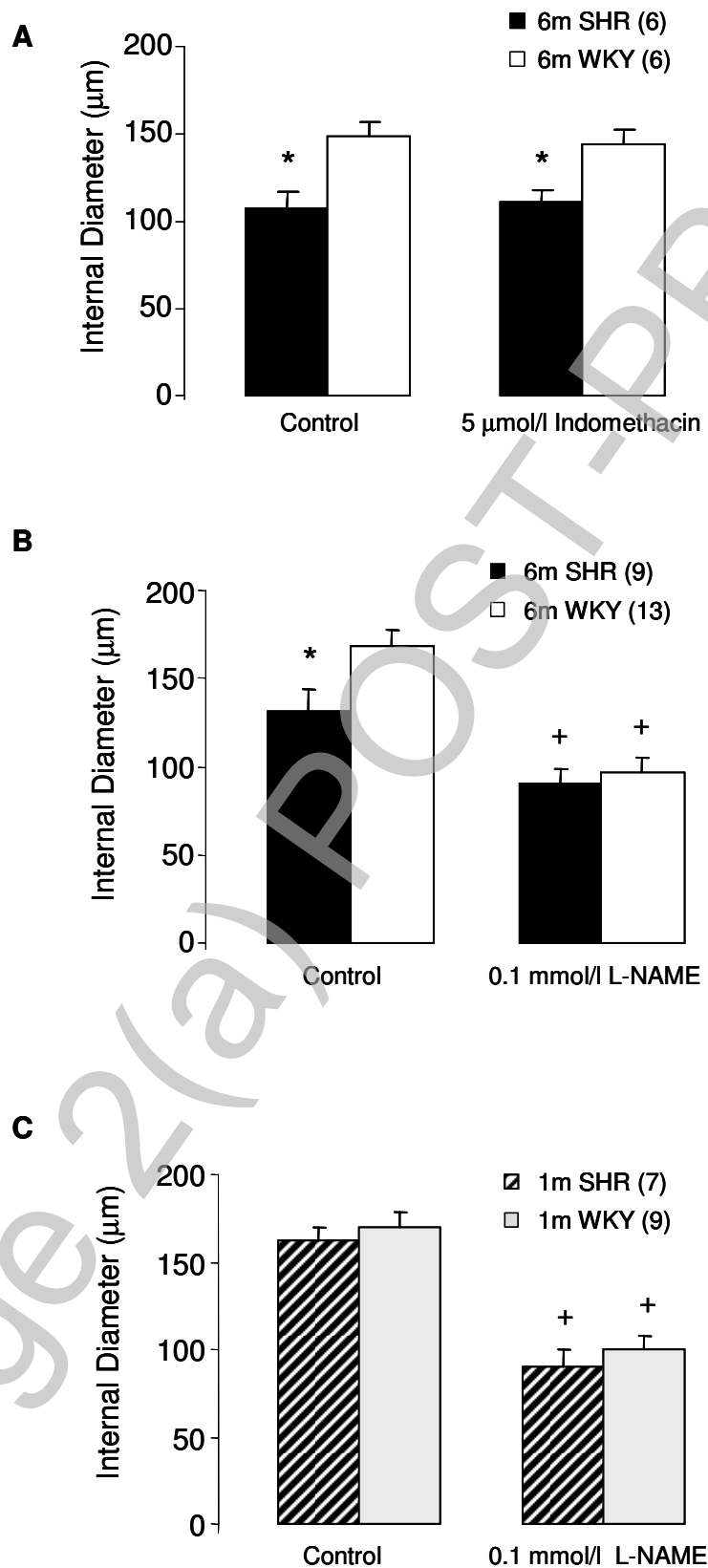


Figure 3

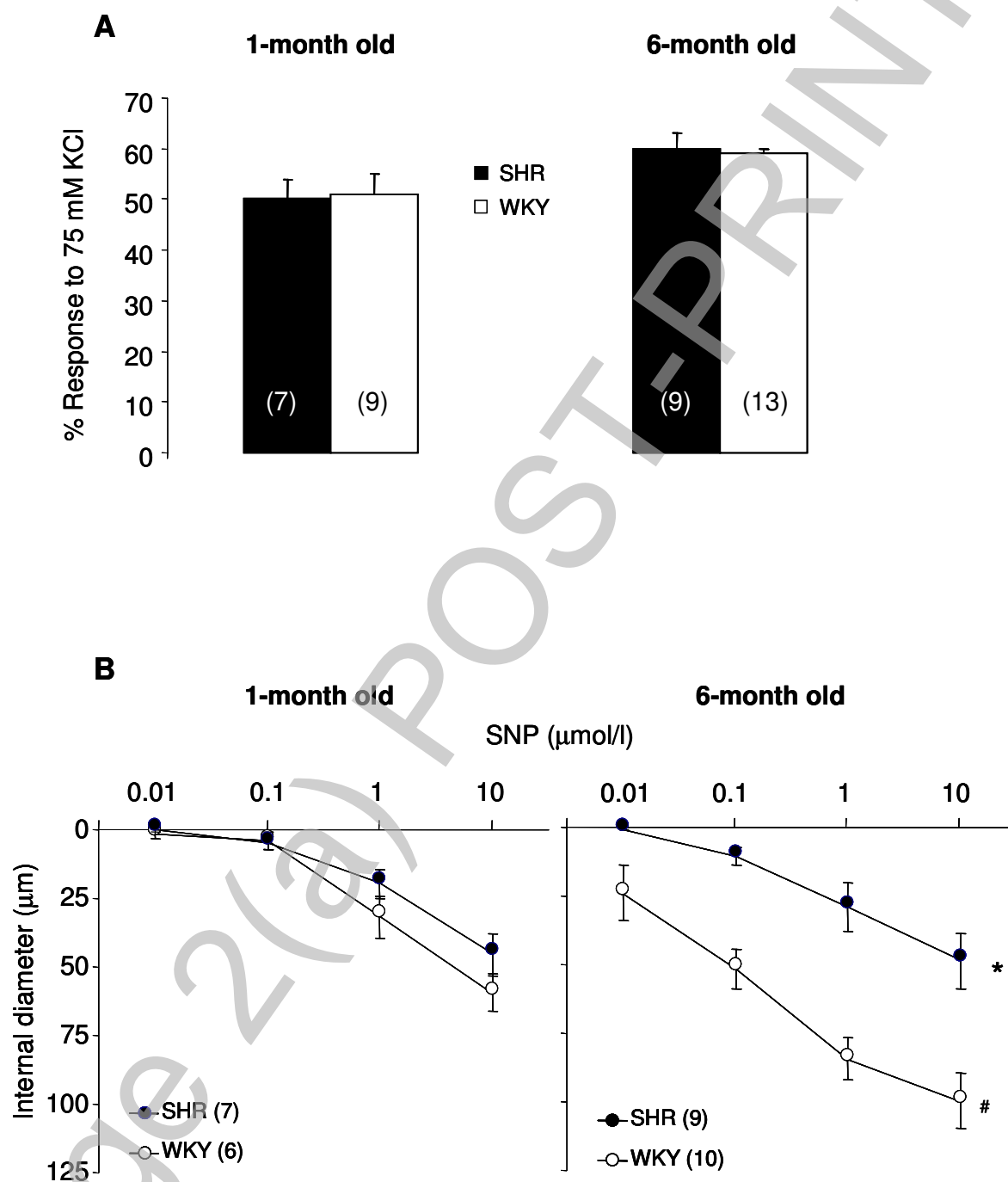


Figure 4

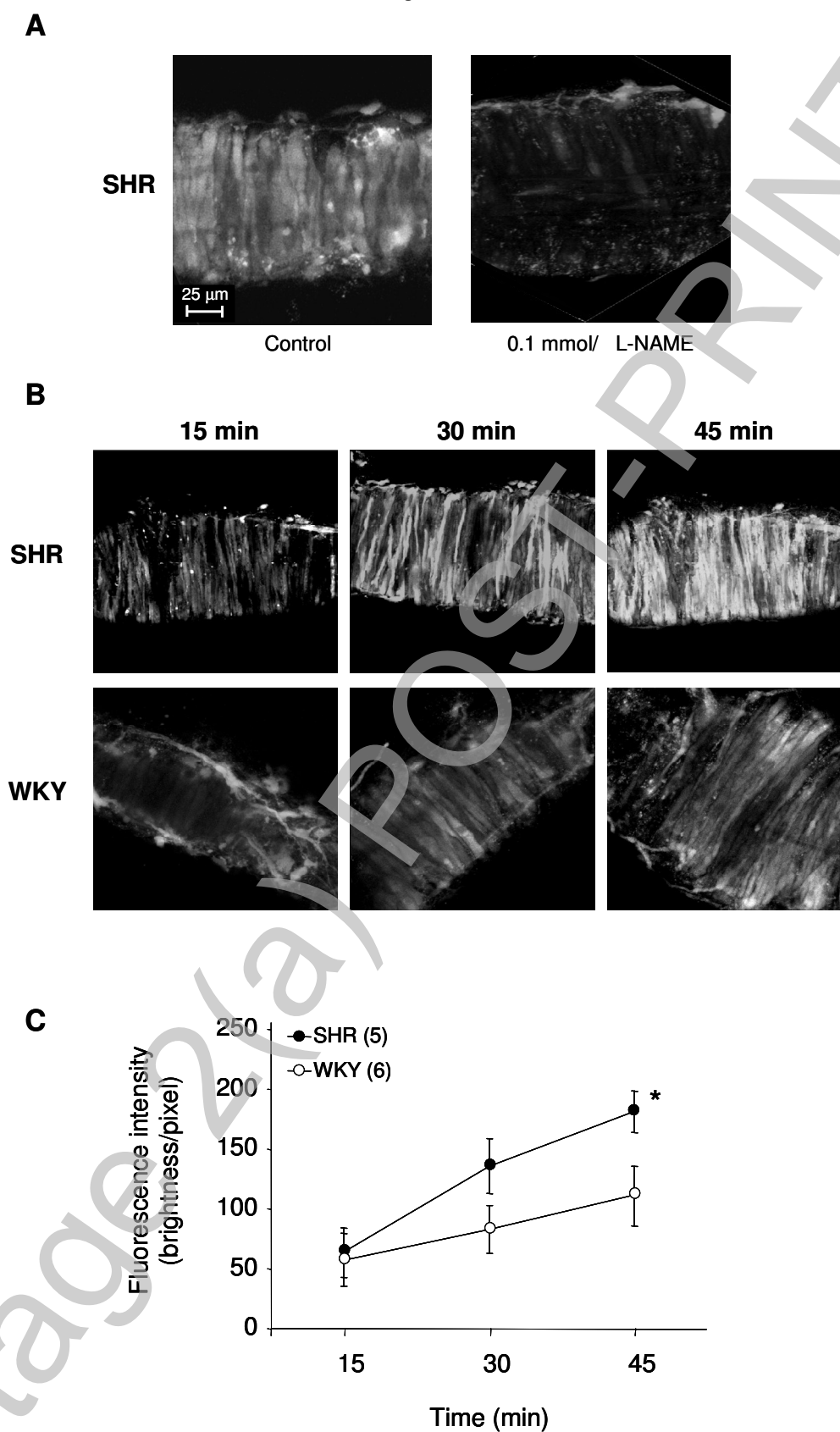


Figure 5

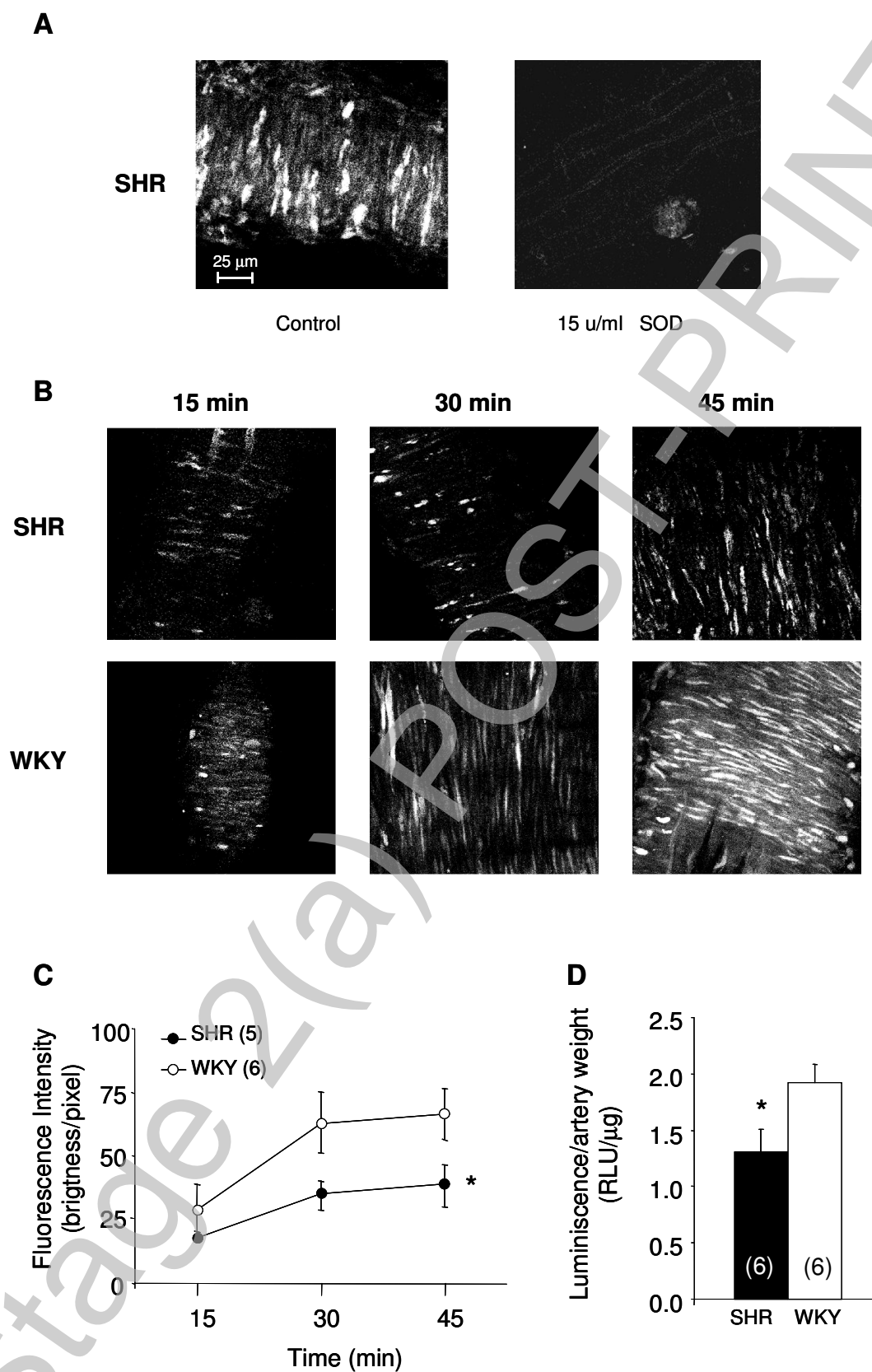


Figure 6

