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c-Src, ERK1/2 and Rho Kinase mediate hydrogen peroxide induced vascular contraction in hypertension. Role of TXA2, NAD(P)H Oxidase and mitochondria

--Manuscript Draft--

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Abstract:	Aim: to analyze the signaling pathways involved in H_2O_2 vascular responses in hypertension. Methods: Vascular function, thromboxane A_2 (TXA_2) production, oxidative stress and protein expression were determined in mesenteric resistance arteries (MRA) from hypertensive (SHR) and normotensive WKY rats. Results: H_2O_2 and the TP agonist U46619 induced greater contractile responses in MRA from SHR than WKY. Moreover H_2O_2increased TXA_2production more in SHR than in WKY. The c-Src inhibitor PP1 reduced H_2O_2- and U46619-induced contraction and TXA_2 release in both strains. The ERK1/2 inhibitor PD98059 reduced H_2O_2but not U46619-induced contraction only in SHR arteries. The Rho Kinase inhibitor Y26372 reduced $H_2O_2$and U46619-induced contractions only in SHR arteries. Basal c-Src, ERK1/2 and Rho Kinase expression were greater in MRA from SHR than WKY. In SHR, the combination of PD98059 with the TP antagonist SQ29548 but not with Y27632, inhibited the H_2O_2contraction more than each inhibitor alone. $H_2O_2$and U46619 increased NAD(P)H Oxidase activity and O_2.- production and decreased mitochondrial membrane potential in vessels from SHR. The effects induced by H_2O_2were abolished by inhibitors of TXA_2synthase, ERK1/2 and c-Src. The mitochondrial antioxidant mitoTEMPO reduced H_2O_2-induced contraction and NAD(P)H Oxidase activation. Conclusions: In arteries from WKY, c-Src mediates H_2O_2 contractile responses by modulating TXA_2release and TXA_2 effect. In SHR, H_2O_2contraction is mediated by c-Src-dependent TXA_2 release which further activates Rho Kinase, c-Src and the relationship between mitochondria and NAD(P)H Oxidase. Moreover, ERK1/2 activation contributes to H_2O_2 contraction in SHR through effects on mitochondria/NAD(P)H Oxidase.

UNIVERSIDAD AUTONOMA DE MADRID

Dr. A. Stella
Executive Editor. *Journal of Hypertension*

April 1st 2014

Dear Editor,

It was a pleasure to learn that our Manuscript JH-D-13-00369, entitled "ROLE OF c-Src, ERK1/2 AND Rho Kinase IN HYDROGEN PEROXIDE INDUCED CONTRACTION IN RESISTANCE ARTERIES FROM NORMOTENSIVE AND HYPERTENSIVE RATS" by García-Redondo et al. was found interesting although a not sufficiently high priority was given. The work has been revised in order to accommodate the Reviewer´s suggestions. New experiments regarding: 1) basal expression of pc-Src, pERK and RhoKinase, 2) involvement of mitochondrial oxidative stress in H_2O_2 responses, 3) the role of TXA_2 in the relationship between mitochondria and NADHPH oxidase, and 4) measurement of ROS production, have been performed as suggested. Accordingly, the manuscript and figures have undergone major changes and the title has been changed. In addition, new authors have been added to the manuscript.

We affirm that the present study is not under consideration by another journal, and that no part of the material has been published elsewhere. All persons acknowledged have seen and approved the mention of their name in the paper. We also state that there is no conflict of interest.

We hope that the new version of the manuscript fulfills all the requirements for its publication in *Journal of Hypertension*. Nevertheless, if anything needs further clarification, please do not hesitate to contact us.

Sincerely yours,

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Answers to Reviewer 1

We would like to thank the Reviewer for his/her constructive comments. The manuscript has undergone changes to include data from new experiments. All concerns raised have been addressed and a detailed list of changes made is attached. We believe that the paper is significantly improved and hope that it is now acceptable for publication.

I have no major concerns regarding this study apart from a couple of considerations:
1) The potential "translational" meaning of the observed findings could be more clearly addressed and detailed.

The Reviewer raised an interesting aspect. It is now evident from an overwhelming amount of experimental and animal studies, that oxidative stress has a role in the etiology of hypertension. Unfortunately, data in patients are more varying and still no clear picture of this aspect has been drawn. Oxidative stress contributes to key features of vascular damage in hypertension, namely endothelial dysfunction, vascular remodeling and inflammation. This might be explained, in hypertensive patients, not only by the increased ROS bioavailability observed systemically or locally (Lacy et al., Hypertension. 2000; 36:878-84), but also by the enhanced redox signaling developed in these conditions (Touyz et al., Hypertension. 2001;38:56-64). Our data further build on this notion demonstrating that different intracellular pathways are involved in H_2O_2 signaling in normotensive and hypertensive animals. Importantly, some of these pathways have a key role in cardiovascular function in pathological conditions. Our data also suggest that increased basal expression/activity of these intracellular pathways might sensitize vessels to H_2O_2 actions. Finally, we demonstrate that oxidative stress is an important perpetuator of key inflammatory pathways including prostanoids and mitochondria which act in relationship to induce vascular dysfunction. How this occurs in hypertension in humans requires further investigations. However, improving our knowledge of H_2O_2 signaling at vascular level particularly in pathological conditions, will help us to develop more specific or more directed strategies to regulate ROS signaling to treat hypertensive vascular damage. Some comments on these aspects have been now added in the revised version of the manuscript.

2) Would the use of a antioxidant (e.g. tempol) provide further information about the mechanism explored ?

In our previous study (García-Redondo et al., J Hypertens. 2009 27(9):1814-22) we demonstrated that treatment of SHR rats with the superoxide analogue Tempol, normalized the increased H_2O_2 contractile responses observed in SHR. Following Reviewer´s recommendations, we have now extended these observations and we have performed new experiments (new Figures 4 and 5) that demonstrate that H_2O_2-derived TXA_2 activates c-Src that participates in mitochondrial ROS production which in turn activates NADPH oxidase and modulate vascular H_2O_2 contraction in small arteries from SHR. To our knowledge, this is the first study that demonstrates that prostanoids derived from H_2O_2 participate in the relationship existing between NADPH oxidase and mitochondria.

Some minor spelling errors may be corrected (e.g. abstract: trough, etc..).

We apologize for the spelling errors. These have been now corrected through the manuscript.

Answers to Reviewer 2

We would like to thank the Reviewer for his/her constructive comments. The manuscript has undergone changes to include data from new experiments. All concerns raised have been addressed and a detailed list of changes made is attached. We believe that the paper is significantly improved and hope that it is now acceptable for publication.

Specific points:

1. The model (Fig. 5) and the conclusions are largely based on the results of the myograph studies. The model would be more convincing if direct evidence for ERK1/2 activation as well as RhoA/ROCK activation could be provided. The study so far showed differential dependence on these signalling mechanisms but not differential activation (e.g. increased phosphorylation of ROCK substrates or ERK1/2 activation).

We agree with the Reviewer that studies on differential activation of the intracellular pathways involved in H_2O_2 responses would have added more convincing evidences. We have tried to perform these experiments by evaluating the effect of H_2O_2 on ERK1/2 and c-Src activation in mesenteric resistance arteries. Unfortunately, we have observed that basal protein activation was very high and we were not able to see further activation in response to H_2O_2. We believe that this might be due to the fact that dissection of the mesenteric resistance arteries takes longer than dissection of other vessels such as aorta and therefore, it is possible that spontaneous kinase activation might be occurring. Most of the articles evaluating intracellular pathways in response to different stimuli usually study effects on isolated cell culture systems (i.e smooth muscle cells or endothelial cells) in which the stimulation conditions are well controlled. In fact, in a previous article Tabet et al., (J Hypertens. 2005 23(11):2005-12) demonstrated that H_2O_2 stimulated phosphorylation of ERK1/2 in cultured vascular smooth muscle cells being this activation greater in SHR than in WKY. We cannot also forget that these intracellular signaling pathways are usually activated within few minutes of stimulation. Therefore, it is not surprising that kinase activation studies are more difficult to perform in whole arteries such as mesenteric resistance arteries that need longer periods of dissection. To try to compensate for these problems, we have evaluated basal expression of the intracellular signaling pathways involved in H_2O_2 responses in mesenteric resistance arteries from normotensive and hypertensive rats. As can be observed in new Figures 1-3, c-Src, ERK1/2 and RhoKinase expression were increased in mesenteric arteries from SHR compared to WKY which might help to explain, at least in part, the increased participation of these intracellular pathways in H_2O_2 and/or TXA_2-induced responses in SHR vessels.

2. Page 8, 1st para: It is somewhat surprising that ROCK inhibition did not affect phenylephrine (PE)-induced vasoconstriction in WKY or SHR vessels while it did affect contraction in response to H2O2 and U46619 at least in SHR. Please document that wall tension in response to PE was the same and stable over the experimentation time with and without ROCK inhibition.

We understand Reviewer´s concerns about the effect of Y27632. As shown in the Table below, there seems to be a tendency to need a slightly higher dose of Phe to achieve approximately 50% of KCl responses, although this difference is not significant. Similarly, Phe responses seem to be slightly smaller in Y277632 incubated arteries that in control vessels, but again did not achieve statistical differences. Therefore, we believe that the effects of Y277632 on H_2O_2 or U46619-induced contractile responses are specifically due to the role of Rho Kinase in the effects of these compounds and only in SHR arteries.

SHR							
Rat	Exp. Condition	Dose Phe* (uM)	Response (%KCl)		Exp. Condition	Dose Phe* (uM)	Response (%KCl)
SHR1	control	1,00	36,53		y27632	3	23,32
SHR2	control	2,00	20,99		y27632	2	81,39
SHR3	control	2,00	54,46		y27632	2	38,05
SHR4	control	2,00	81,55		y27632	2	43,73
SHR5	control	2,00	60,34		y27632	3	17,78
SHR6	control	2,00	64,61				
SHR7	control	3,00	19,76				
	Mean	2,00	48,32			2,40	40,85
	SEM	0,2	8,8			0,2	11,2
						t-test vs control	t-test vs control
						0,254981145	0,606586138
		Dose Phe* indicates Phe needed to achieve a contractile response of aprox 50% of KCl response.					

3. NADPH oxidase assay: Why was H2O2 added in cumulative doses? Did you obtain a full concentration response curve for NADPH-dependent superoxide formation? What was the H2O2 concentration for the data presented in Fig. 4?

We apologize for the lack of clarity. We did not obtain a full concentration response curve for NADPH-dependent superoxide formation. The reason of adding H_2O_2 in cumulative doses was just to add H_2O_2 in the same way that was added to the organ bath for the contractile contraction response curves. Therefore, the final concentration of H_2O_2 in the samples was 100 µM. This aspect has been now clarified in the methods section (page 9, first paragraph).

4. Although previous data of the authors showed low rates of superoxide formation in WKY-vessels and no effect of antioxidants on H2O2-induced vasoconstriction, it should be confirmed that H2O2 does not activate NADPH oxidase activity in WKY vessels.

Following Reviewer´s suggestion we have performed new experiments to evaluate NADPH oxidase activity in WKY vessels in response to H_2O_2. Interestingly, in MRA from WKY rats, H_2O_2 also activated NAD(P)H Oxidase. These results are now shown in the manuscript (Page 14, second paragraph). This and our previously published results (García-Redondo et al., J Pharmacol Exp Ther. 2009;328:19-27; García-Redondo et al., J Hypertens. 2009;27:1814-22) suggest that although H_2O_2 activates NAD(P)H Oxidase in

arteries from normotensive animals, this does not have a role in H_2O_2-induced contraction.

5. *Page 11, 1st paragraph: The authors compare data obtained with SQ29548, PD98059 and the combination of the two. The data were obviously obtained in separate experiments (Fig. 1 SQ29548 alone, the other two in Fig. 2) with quantitatively different responses in the control groups. How were between experiment comparisons performed (statistics)?*

The Reviewer is right. For all the figures, the different experimental conditions were performed in parallel in separate segments. Thus, for each inhibitor to be tested, control and drug-incubated segments from the same animal were used. This is the reason explaining why the controls are slightly different from one figure to the other. For Figure 2, our experimental set up was also the same. Thus, in the same experiment (rat) we had Control, PD98059-, SQ29548- and PD98059+SQ29548-incubated segments. In the previous version of the manuscript, we had omitted the SQ29548 data to avoid redundancy with data in Figure 1. We have now added the respective data of SQ29548-incubated arteries in Figure 2.

Page 11, 2nd paragraph: The authors report that the responses to Y27632 and Y27632+SQ29548 were not different (results section and abstract). However, at the highest H2O2 concentration the contraction is about 20-30% less in the combined group than in the Y27632 only group (SHR vessels). The control group shown in Fig. 3 appears to be the same as in Fig 1.

Although there seems to be less contraction in the combined group, neither one-way ANOVA nor individual Bonferroni t-test at this dose give statistical significance.
Yes, the control group in Figure 3 is the same as in Figure 1 because from each animal we were able to test different inhibitors (we can mount up to 12 different arteries from one animal) and therefore the control would be the same for both inhibitors. The fact that in other Figures the controls are different is because not all protocols could be tested in the same animals and different animals were used thereafter. As mentioned above, in each animal there was always the control situation.

The concentration response curves for U46619 (controls, filled squares) appear to be the same in Figs. 1-3 although the numbers of individuals vary (SHR panels). Please explain how the study was organized to allow for proper comparison and analysis for effects of rat strain and treatment (statistics).

The Reviewer is right. The same concentration curve for U46619 is displayed in Figures 1-3 and all the inhibitors were evaluated in the same animals therefore having the same controls. We apologize for the number of animals in Figure 3. This was a mistake. The correct number is 6 for both figures. This has been now corrected in the new version of the manuscript.

Minor

Page 7: Please indicate the phenylephrine concentration used for precontraction.

The concentration of Phenylephrine used for precontraction was that to achieve approximately 50% of maximal response. This has been now added in the revised version of the manuscript (Page 8, line 1).

Page 10, line 6 from bottom: PPI reduced (not normalized)? the H2O2-induced TXA2 release.

We thank the Reviewer to point out this aspect. However, we believe that the term *normalized* is correct since as can be observed in Figure 1 PP1 incubation decreased TXB_2 levels to reach those observed in control situation both in WKY and in SHR.

Answers to Reviewer 3

We would like to thank the Reviewer for his/her constructive comments. The manuscript has undergone changes to include data from new experiments. All concerns raised have been addressed and a detailed list of changes made is attached. We believe that the paper is significantly improved and hope that it is now acceptable for publication.

Major concerns

1. Although the study is well-conducted and the key findings of the present study as well as differences between the vascular responses in SHR and WKY are clear and nicely demonstrated, the study somehow suffers just from being an extension of their previous study and the data are to some extent confirmatory. In their previous studies the authors have already demonstrated the strain-related difference in H2O2-induced contractile responses, and on the other hand, the involvement of c-Src, ERK1/2 and Rho kinases in the pathogenesis of vascular dysfunction with hypertension has already been demonstrated in several previous studies. Therefore the novelty of the present study remains unfortunately rather limited.

We understand Reviewer´s concerns. It is true that in our previous studies we have demonstrated that H_2O_2 contraction and basal and H_2O_2 -stimulated TXA_2 production were greater in SHR mesenteric arteries compared to WKY (García-Redondo et al., J Pharmacol Exp Ther. 2009;328:19-27; García-Redondo et al., J Hypertens. 2009;27:1814-22). It is important to emphasize that we needed to repeat these experimental protocols in order to deeply analyze the underlying intracellular mechanisms responsible of H_2O_2 actions and this was no evaluated in any of our previous studies. It is also true that many papers have demonstrated the key role of c-Src, ERK1/2 and Rho kinase in vascular dysfunction in hypertension. However, to our knowledge very few papers have demonstrated specifically the intracellular pathways responsible of H_2O_2 responses in hypertension in whole arteries. In this sense, it is important to note that most of the articles evaluating intracellular pathways in hypertensive vascular responses have been performed in isolated cultured vascular cells or in response to other stimuli such as Angiotensin II.

Following Reviewer´s suggestion we have performed new experiments in order to enhance the novelty of the manuscript. We have therefore focused our efforts in evaluating the role of mitochondrial oxidative stress in some of the observed effects. This is an important aspect since recent studies demonstrate the key role of H_2O_2 and mitochondria in vascular dysfunction in Ang II-induced hypertension (for review see Dikalov and Ungvari Z. Am J Physiol Heart Circ Physiol. 2013;305:H1417-27), although to our knowledge no studies on this regard have been performed in a model of essential hypertension such as the SHR. Herein we demonstrate for the first time, that prostanoids particularly TXA_2, are able to activate the relationship existing between NADPH oxidase and mitochondria in hypertension, that this has a role in vascular dysfunction induced by H_2O_2 and that c-Src has a key role in these effects. These aspects have been properly discussed throughout the manuscript.

2. The authors should consider analyzing the expression (total and phosphorylated forms by Western blot) of their target proteins, and if possible vascular superoxide production.

We agree with the Reviewers 3 and 2 that studies on differential activation of the intracellular pathways involved in H_2O_2 responses would have added more convincing evidences. We have tried to perform these experiments by evaluating the effect of H_2O_2 on ERK1/2 and c-Src activation in mesenteric resistance arteries. Unfortunately, we have observed that basal protein activation was very high and we were not able to see further activation in response to H_2O_2. We believe that this might be due to the fact that dissection of the mesenteric resistance arteries takes longer than dissection of other vessels such as aorta and therefore, it is possible that spontaneous kinase activation might be occurring. Most of the articles evaluating intracellular pathways in response to different stimuli usually study effects on isolated cell cultured systems (i.e smooth muscle cells or endothelial cells) in which the stimulation conditions are well controlled. In fact, in a previous article Tabet et al., (J Hypertens. 2005 23(11):2005-12) demonstrated that H_2O_2 stimulated phosphorylation of ERK1/2 in cultured vascular smooth muscle cells being this activation greater in SHR than in WKY. We cannot also forget that these intracellular signaling pathways are usually activated within few minutes of stimulation. Therefore, it is not surprising that kinase activation studies are more difficult to perform in whole arteries such as mesenteric resistance arteries that need longer periods of dissection. To try to compensate for these problems, we have evaluated basal expression of the intracellular signaling pathways involved in H_2O_2 responses in mesenteric resistance arteries from normotensive and hypertensive rats. As can be observed in new Figures 1-3, c-Src, ERK1/2 and RhoKinase expression were increased in mesenteric arteries from SHR compared to WKY which might help to explain, at least in part, the increased participation of these intracellular pathways in H_2O_2 and/or TXA_2-induced responses in SHR vessels.

As suggested by the Reviewer, we have also determined vascular $O_2^{\cdot -}$ production in SHR mesenteric vessels. As shown in new Figure 4, H_2O_2 increased $O_2^{\cdot -}$ production that was abolished by furegrelate, U0126 and PPI. This would be in agreement with the effects of these inhibitors on NADPH Oxidase activity.

This article examines the signaling pathways involved in H_2O_2 vascular responses in hypertension. Our results demonstrate increased vasoconstrictor responses to H_2O_2 and the TP analogue U46619 and increased TXA_2 production in arteries from hypertensive (SHR) than normotensive (WKY) rats. In arteries from WKY, c-Src mediates H_2O_2 contractile responses by modulating TXA_2 release and TXA_2 effect. In SHR, H_2O_2 contraction is mediated by c-Src-dependent TXA_2 release which further activates Rho Kinase, c-Src and the relationship between mitochondria and NAD(P)H Oxidase. Moreover, ERK1/2 activation contributes to H_2O_2 contraction in SHR through effects on mitochondria/NAD(P)H Oxidase.

List of non-standard abbreviations: ROS: reactive oxygen species; H_2O_2: hydrogen peroxide; MAPKs: mitogen activated protein kinases; $O_2^{\cdot-}$: superoxide anion; TXA_2: thromboxane A_2; VSMC: vascular smooth muscle cells; MRA: mesenteric resistance arteries; SHR: spontaneously hypertensive rats; WKY: Wistar Kyoto; KHS: Krebs Henseleit solution.

c-Src, ERK1/2 and Rho Kinase mediate hydrogen peroxide induced vascular contraction in hypertension. Role of TXA_2, NAD(P)H Oxidase and mitochondria

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Short title: signaling pathways in H_2O_2 vasoconstrictor responses

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Conflicts of interest: None declared

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Abstract

Aim: to analyze the signaling pathways involved in H_2O_2 vascular responses in hypertension. Methods: Vascular function, thromboxane A_2 (TXA_2) production, oxidative stress and protein expression were determined in mesenteric resistance arteries (MRA) from hypertensive (SHR) and normotensive WKY rats. Results: H_2O_2 and the TP agonist U46619 induced greater contractile responses in MRA from SHR than WKY. Moreover H_2O_2 increased TXA_2 production more in SHR than in WKY. The c-Src inhibitor PP1 reduced H_2O_2- and U46619-induced contraction and TXA_2 release in both strains. The ERK1/2 inhibitor PD98059 reduced H_2O_2 but not U46619-induced contraction only in SHR arteries. The Rho Kinase inhibitor Y26372 reduced H_2O_2 and U46619-induced contractions only in SHR arteries. Basal c-Src, ERK1/2 and Rho Kinase expression were greater in MRA from SHR than WKY. In SHR, the combination of PD98059 with the TP antagonist SQ29548 but not with Y27632, inhibited the H_2O_2 contraction more than each inhibitor alone. H_2O_2 and U46619 increased NAD(P)H Oxidase activity and $O_2^{\cdot -}$ production and decreased mitochondrial membrane potential in vessels from SHR. The effects induced by H_2O_2 were abolished by inhibitors of TXA_2 synthase, ERK1/2 and c-Src. The mitochondrial antioxidant mitoTEMPO reduced H_2O_2-induced contraction and NAD(P)H Oxidase activation. Conclusions: In arteries from WKY, c-Src mediates H_2O_2 contractile responses by modulating TXA_2 release and TXA_2 effect. In SHR, H_2O_2 contraction is mediated by c-Src-dependent TXA_2 release which further activates Rho Kinase, c-Src and the relationship between mitochondria and NAD(P)H Oxidase. Moreover, ERK1/2 activation contributes to H_2O_2 contraction in SHR through effects on mitochondria/NAD(P)H Oxidase.

Keywords: c-Src, ERK1/2, Rho Kinase, NAD(P)H Oxidase, mitochondria, H_2O_2, SHR, mesenteric resistance arteries

Introduction

Hydrogen peroxide (H_2O_2) is a cell-permeant and highly stable reactive oxygen specie (ROS) that is produced mainly by the dismutation of superoxide anion ($O_2^{\cdot-}$) by superoxide dismutase and also directly by the NOX-4 isoform of the NAD(P)H Oxidase [1,2]. H_2O_2 levels are tightly regulated by intracellular and extracellular enzymes including catalase, glutathione peroxidase, thioredoxin, and other peroxyredoxins, which convert H_2O_2 to water and O_2 [1,3]. It is now accepted that the vascular functional effects of H_2O_2 are very complex and depend among other factors, of the specific vascular bed studied and the nature of the pre-contractile agent. Thus, H_2O_2 can either contract or relax arteries from different species in resting state or after preconstriction with stimuli like phenylephrine, high K^+ solution or others [1,4-7].

The relationship between hypertension and the increased production of ROS has been extensively demonstrated in experimental models of hypertension and in humans [3]. Thus, increased levels of H_2O_2 have been described in plasma [8] and tissues [9] from hypertensive patients. More importantly, H_2O_2 induces greater contractile responses in conductance and resistance vessels from hypertensive than normotensive animals [4,6,7,10,11] further promoting vascular dysfunction. Two of the major ROS sources at vascular level include NAD(P)H Oxidase and mitochondria [2, 12]. Interestingly, in the past few years a cross talk between mitochondria and NAD(P)H oxidase has been demonstrated in the setting of hypertension with H_2O_2 having a pivotal role [12]. This relationship is particularly evident for Angiotensin II stimulation and seems to have a role in vascular function alterations [12-15]. However, whether this is also present in essential hypertension is less known.

Among the mechanisms whereby H_2O_2 induces vasoconstriction, stimulation of cyclooxygenase and release of the vasoconstrictor TXA_2 appear to be pivotal [4-6,16].

Several studies have demonstrated the role of c-Src, ERK1/2 mitogen activated protein kinases (MAPKs) and Rho Kinase in the contractile effects of H_2O_2 or the TXA_2 analogue U46619 in different vessels from mice or rat [17-22]. In addition, in cultured vascular smooth muscle cells (VSMC) the activation of ERK1/2 induced by H_2O_2 is greater in spontaneously hypertensive (SHR) than in normotensive Wistar Kyoto rats (WKY) [23]. However, the specific intracellular pathways involved in H_2O_2-induced TXA_2 release or whether these intracellular pathways activated by H_2O_2 and/or TXA_2 are different in vessels from hypertensive animals and participate in vascular function, is unknown.

We have previously demonstrated that H_2O_2 induces greater TXA_2 release and contractile responses in mesenteric resistance arteries (MRA) from SHR than WKY [6,7]. In addition, in SHR arteries, the contractile effect of H_2O_2-derived TXA_2 is partially mediated by $O_2^{\cdot-}$ production [6,7]. In the present study we aimed to analyze the intracellular signalling pathways involved in the effects of H_2O_2 in arteries from WKY and SHR and the role of NAD(P)H Oxidase and mitochondrial oxidative stress in these effects.

Methods

Animals

6 month-old male SHR and WKY rats were obtained from colonies maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid. The investigation conforms to the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the current Spanish and European laws (RD 223/88 MAPA and 609/86). Rats were euthanized by decapitation and the mesenteric vascular bed and the aorta

were removed and placed in cold (4°C) Krebs–Henseleit solution (KHS) (in mmol/L: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose and 0.01 Na_2EDTA) bubbled with a 95% O_2–5% CO_2 mixture. Segments of second and third order branches of the superior mesenteric artery and aorta were dissected free of fat and connective tissue.

Reactivity experiments

Third order branches of the mesenteric artery, 2 mm in length, were mounted in a small vessel dual chamber myograph for measurement of isometric tension. Two steel wires (40 μm diameter) were introduced through the lumen of the segments and mounted according to the method described by Mulvany and Halpern [24]. After a 30 min equilibration period in oxygenated KHS at 37 °C and pH = 7.4, segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-wall tension ratio of the segments by setting their internal circumference, Lo, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg [20 24]. Segments were washed with KHS and left to equilibrate for 30 min; segment contractility was then tested by an initial exposure to a high K^+ solution (120 mmol/L K^+-KHS, which was identical to KHS except that NaCl was replaced by KCl on an equimolar basis). The maximum response to K^+-KHS was similar in arteries from both strains (WKY: 2.8 ± 0.2 mN/mm, n=12 SHR: 2.9± 0.3 mN/mm, n=15). The presence of endothelium was determined by the ability of 10 μmol/L acetylcholine to induce relaxation in arteries precontracted with phenylephrine to achieve a contractile response of approximately 50% of maximum response.

In previous experiments, we observed that H_2O_2 does not induce vascular responses in quiescent mesenteric arteries. We therefore performed the experiments in

phenylephrine-contracted vessels at a dose that produced ~ 50% K+-KHS contraction, as described [6]. Once the presence of endothelium was determined, a cumulative concentration-response curve to H_2O_2 (1-100 μmol/L) or U46619 (0.1 nmol/L-1 μmol/L) was performed in segments contracted with phenylephrine as described above. The participation of different signalling pathways was analysed by preincubation of the arteries with PD98059, an ERK1/2 MAPK inhibitor, Y27632, a Rho Kinase inhibitor, PP1, a c-Src inhibitor or mito-TEMPO, a mitochondrial targeted superoxide dismutase mimetic. In another set of experiments, arteries were incubated with the TP antagonist SQ29548 alone or in combination with PD98059 or with Y27632. All drugs were added 30 min before the H_2O_2 or U46619 concentration-response curve and none of the inhibitors modified phenylephrine vasoconstriction. At the end of the experiment, the viability of the vessels was tested by re-exposure to K^+-KHS.

Measurement of TXA_2 production

Second and third order branches of the mesenteric artery from WKY and SHR were incubated in oxygenated KHS (37ºC). After 30 min stabilization, phenylephrine (1 μmol/L) was added for 4 min. Thereafter, cumulative doses of H_2O_2 (1-100 μmol/L, 4 min each dose) were added to the incubation medium to mimic the same conditions to those found in the organ bath. The participation of c-Src on TXA_2 release was evaluated by preincubation of the arteries with PP1 30 min before phenylephrine. At the end, the medium was collected and frozen (-80ºC) for further analysis. The levels of the metabolite of TXA_2, TXB_2, were determined using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Harbor, Mi, USA). The levels of TXB_2 were normalized per μg of protein.

NAD(P)H Oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine the NAD(P)H Oxidase activity in mesenteric homogenates. Second and third order branches of the mesenteric artery from SHR and WKY were incubated in oxygenated KHS (37ºC). After 30 min stabilization, phenylephrine (1 µmol/L) was added for 4 min. Thereafter, cumulative doses of H_2O_2 were added to the incubation medium to mimic the same experimental conditions that those found in the organ bath. Final concentration of H_2O_2 was 100 µmol/L. In SHR vessels the participation of TXA_2 synthase, ERK1/2, Rho Kinase and c-Src on NAD(P)H Oxidase activity was evaluated by preincubation with the respective inhibitors furegrelate, U0126 (another ERK1/2 MAPK inhibitor), Y27632 and PP1, that were added 30 min before phenylephrine. In another set of experiments, aortic segments were incubated with U46619 (10 µmol/L) in the absence or in the presence of PP1, or with H_2O_2 in the absence or in the presence of mito-TEMPO or the inhibitor of the complex I of the electron transport chain, rotenone. At the end, tissues were homogenized in lysis buffer (in mmol/L: KH_2PO_4, 50; ethyleneglycoltetraacetic acid, 1; sucrose 150; pH=7.4). The reaction was started by the addition of NAD(P)H (0.1 mmol/L) to the suspension containing sample, lucigenin (5 µmol/L) and assay phosphate buffer. The luminescence was measured in a plate luminometer (AutoLumat LB 953, Berthold, Germany). Buffer blank was subtracted from each reading. Activity is expressed as relative light units/mg protein and variations of NAD(P)H Oxidase activity were calculated as percentage of control situation.

In situ detection of vascular $O_2^{\cdot-}$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate $O_2^{\cdot-}$ production *in situ*, as previously described [7]. Third order branches of the mesenteric artery from SHR were incubated in oxygenated KHS (37ºC). After 30 min stabilization, arteries were stimulated with H_2O_2 in the absence or in the presence of furegrelate,

U0126 or PP1, as described above. Then arterial segments were placed in PBS containing 30% sucrose for 20 min, transferred to a cryomold containing Tissue Tek OCT-embedding medium, and frozen in liquid nitrogen. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (in mmol/l: 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, 11 glucose, pH=7.4). Fresh buffer containing DHE (2 µmol/l) was topically applied onto each tissue section, cover-slipped, incubated for 30 min in a light-protected humidified chamber at 37°C and then viewed with a fluorescent laser scanning confocal microscope (Leica TCS SP2 equipped with a krypton/argon laser, x40 objective). Every day, control and H_2O_2-incubated arteries in the absence or in the presence of the different inhibitors were imaged in parallel using the same imaging settings. Fluorescence was detected with a 568 nm long-pass filter. For quantification, two-three rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated. To minimize laser fluctuations from one day to another, data were expressed as % of signal in control arteries.

Mitochondrial Membrane Potential (ΔΨ)

Mitochondrial membrane potential was monitored with the fluorescent probe tetramethylrhodamine methyl ester (TMRE, Ex/Em, 543/573 nm) (Life Technologies). Second and third order branches of the mesenteric artery or aortic segments from SHR were incubated in oxygenated Krebs-HEPES buffer (37ºC). After 30 min stabilization, arteries were stimulated with H_2O_2 or with U 46619 in the absence or in the presence of furegrelate, U0126 or PP1 as described above. Thereafter, arteries were incubated with Krebs-HEPES buffer containing TMRE (0.1 µmol/L) 20 min, 37ºC. After washing, fluorescence intensity was measured with a spectrofluorimeter (FLUOstar OPTIMA

BMG Labtech). Fluorescence intensity values were normalized by the amount of proteins and values were expressed as a % of control arteries.

Western blot analysis

MRA from WKY and SHR rats were dissected free of fat and connective tissue and frozen at -80ºC. Protein expression was determined in homogenates as previously described [6]. Briefly, proteins (30 μg) were separated by SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight. Membranes were incubated overnight with rabbit or mouse primary antibodies for Rho kinase (1:1000 Santa Cruz Biotechnology, Santa Cruz, California, USA), c-Src (1:1000 Santa Cruz Biotechnology), p-Tyr^{418}-c-Src (1:1000 Biosource International, Inc USA), p-ERK1/2 (1:1000) and GADPH (1: 5000 Sigma Chemical, Co., St Louis, Mi, USA). After washing, membranes were incubated with anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (1:5000, Bio-Rad, Laboratories, Hercules, CA, USA). The immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemiluminiscence system (ECL Plus, Amersham International; Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International). Signals on the immunoblot were quantified using the Quantity One version 4.6.9 computer program.

Drugs and solutions

H_2O_2, acetylcholine, furegrelate, phenylephrine, 9, 11-di-deoxy-11α 9α-epoxymethano prostaglandin F2α (U46619) and rotenone, were obtained from Sigma Chemical Co. SQ29548 was obtained from ICN Iberica, (Barcelona, Spain). PP1, Y27632 and PD98059 were obtained from Biomol (Plymouth Meeting, PA, USA). U0126 was obtained from Calbiochem (San Diego, CA). mito-TEMPO was obtained from Santa

Cruz Biotechnology Inc. U46619 was dissolved in ethanol, PP1 and PD98059 were dissolved in DMSO. Further dilutions were in distilled water.

Data analysis and statistics

Contractile responses for each dose are expressed as a percentage of contraction to K^+-KHS. All data are expressed as mean values ± standard error of the mean (SEM). Results were analyzed by Student's *t*-test, one-way or two-way ANOVA using GraphPad Prism Software (San Diego, CA, USA). A *P* value below 0.05 was considered significant.

Results

As previously described [6], H_2O_2-induced contraction (1-100 μmol/L) was greater in MRA from SHR than WKY rats (Maximum response: WKY: 8.4±2.5, n=9; SHR: 39.1±6.8 %, n=10; $p<0.01$). In addition, U-46619-induced contraction (0.1 nM-1 μM) was also greater in arteries from SHR than WKY (Maximum response: WKY: 57.9±3.5, n=7; SHR: 69.4±3.2 %, n=6; $p<0.05$).

Role of c-Src in H_2O_2 contraction

Previous studies have demonstrated a role of c-Src in mediating H_2O_2-induced contraction in normotensive animals [19]. The c-Src inhibitor PPI (1 μmol/L) abolished the H_2O_2-induced contractile response in MRA from WKY and reduced H_2O_2 responses in SHR (Figure 1A). As previously described [6] the TP antagonist, SQ29548 (1 μmol/L) also abolished H_2O_2-contractile response in MRA from WKY and reduced it in SHR (Figure 1A). PP1 also decreased U46619-induced contractile responses both in WKY and SHR mesenteric arteries (Figure 1B). In agreement with our previous report [6], basal TXA_2 was greater in SHR than WKY and H_2O_2 induced TXA_2 release in both strains reaching higher levels in SHR compared to WKY (Figure 1C). PPI normalized

the H_2O_2-induced TXA_2 release of mesenteric arteries from WKY and SHR (Figure 1C). Additionally, increased basal activation of c-Src was observed in MRA from SHR compared to WKY (Figure 1D). All together, our results demonstrate that in MRA from WKY and SHR, c-Src mediates H_2O_2-induced TXA_2 release and TXA_2 contractile effects. However, additional mechanisms might be involved in H_2O_2 contractile responses in SHR.

Role of ERK1/2 in H_2O_2 contraction

Another important intracellular pathway activated by H_2O_2 is ERK1/2 MAPK [18,19,22,23]. Then, we evaluated the involvement of this kinase in H_2O_2 contraction by using the specific inhibitor PD98059 (10 µmol/L). As shown in Figure 2A, PD98059 inhibited H_2O_2-induced contraction in MRAs from SHR but not from WKY. In addition, PD98059 did not affect U46619-induced contractile responses neither in WKY nor in SHR (Figure 2B). The combination of SQ29548 and PD98059 in arteries from SHR induced a greater inhibitory effect of H_2O_2 contraction than PD98059 and SQ29548 alone (Figure 2A) and almost abolished H_2O_2-induced contraction. In addition, basal ERK1/2 activation was greater in MRA from SHR than WKY (Figure 2C). These results suggest that activation of ERK1/2 by H_2O_2 independent of TP activation might be also responsible of the vascular contraction in SHR.

Role of Rho Kinase in H_2O_2 contraction

It has been suggested that excessive activation of the RhoA/Rho-Kinase pathway might participate in the alteration of the vasoconstrictor responses observed in cardiovascular diseases including hypertension [25]. As shown in Figure 3A, the inhibitor of Rho Kinase Y27632 (1 µmol/L) inhibited H_2O_2-induced contraction in SHR arteries but not in WKY. Similarly, Y27632 inhibited U46619-induced contraction in SHR but not in WKY (Figure 3B). We then combined SQ29548 and Y27632 in arteries from SHR. The

combination of both drugs induced similar effects to that produced by Y27632 (Figure 3A) or SQ29548 (P>0.05) alone. In addition, Rho kinase expression was greater in MRA from SHR than WKY (Figure 3C). This suggests that the participation of Rho Kinase in H_2O_2 effect in SHR might be mediated *via* TXA_2.

H_2O_2 induces NAD(P)H Oxidase activation. Mechanisms involved

We have previously described that H_2O_2-induced vasoconstriction in MRA from SHR but not from WKY is dependent of TXA_2-derived $O_2^{\cdot-}$ production [6,7]. Herein we aimed to determine whether H_2O_2 might activate NAD(P)H Oxidase activity in SHR and the possible mechanisms involved. As shown in Figure 4A, H_2O_2 induces NAD(P)H Oxidase activation in MRA from SHR. This activation was inhibited by the TXA synthase inhibitor furegrelate (1 µmol/L), by PP1, by the ERK1/2 inhibitor, UO126 (10 µmol/L), but not by the Rho Kinase inhibitor Y27632. Accordingly, $O_2^{\cdot-}$ was produced in MRA from SHR in response to H_2O_2, and this production was reduced by furegrelate, PP1, or UO126 (Figure 4B). In addition, in aorta from SHR U46619 increased NAD(P) Oxidase activity and this increase was prevented by PP1. Interestingly, in MRA from WKY rats, H_2O_2 also activates NAD(P)H Oxidase (% Control: 154 ± 5.6 n=6). All together, these results suggest that TXA_2 plays a central role in the activation of NAD(P)H Oxidase by H_2O_2 in SHR. In addition, this activation is tightly regulated by c-Src and ERK1/2.

A cross talk between mitochondria and NAD(P)H oxidase has been demonstrated in hypertension with H_2O_2 having a pivotal role [12]. We therefore aimed to evaluate the role of mitochondrial oxidative stress in H_2O_2 effects. As shown in Figure 5A, the mitochondrial targeted superoxide dismutase mimetic mito-TEMPO (0.5 µmol/L), inhibited H_2O_2-induced contraction in SHR arteries. Moreover, both mito-TEMPO and the inhibitor of mitochondrial respiratory chain rotenone (5 µmol/L), abolished the

H_2O_2-induced NAD(P)H Oxidase activation in aorta from SHR (Figure 5B). Mitochondrial membrane potential ($\Delta\Psi$) is essential for normal mitochondrial function. Treatment of MRA with H_2O_2 caused partial depolarization of mitochondria (Figure 5C) and this effect was blunted by furegrelate, U0126 or PP1. H_2O_2 effect on membrane potential was mimicked in aorta from SHR by U46619 (Figure 5D). The decrease in membrane potential induced by U46619 was prevented by pretreatment with PP1 (Figure 5D). These results suggest that H_2O_2-derived TXA_2 induces mitochondrial dysfunction that participates in H_2O_2 vascular effects in hypertension.

Discussion

The results of the present study demonstrate that different intracellular signalling pathways are responsible of H_2O_2 contraction in MRA from SHR and WKY. In arteries from WKY, c-Src mediates H_2O_2 contractile responses by modulating TXA_2 release and TXA_2 effect. In SHR, H_2O_2 contraction is mediated by c-Src-dependent TXA_2 release which further activates Rho Kinase, c-Src and the relationship between mitochondria and NAD(P)H Oxidase. Moreover, TXA_2-independent activation of ERK1/2 contributes to H_2O_2 contraction in SHR through effects on mitochondria/NAD(P)H Oxidase (Figure 6). All together, these mediators would explain the increased contractile response induced by H_2O_2 in hypertension.

Role of c-Src

The Src family of nonreceptor tyrosine kinases is considered prime targets for redox regulation [21]. The Src family has been implicated in signal transduction of constrictor responses to different stimuli including Angiotensin II and prostanoids like $PGF_{2\alpha}$, which appear to be mediated at least in part, *via* ROS [21]. In addition, c-Src is activated by exogenous H_2O_2 in various cell types including VSMC and endothelial cells [23, 26-28], it mediates H_2O_2 contractile responses [19] but not U46619

contractile responses [19] in aorta from normotensive animals and it is more activated in cultured VSMC from SHR compared to WKY [29]. Herein we demonstrate that c-Src mediates H_2O_2 contractile responses in MRA from normotensive and hypertensive rats. The increased basal activation of this kinase observed in vessels from SHR might contribute to the increased H_2O_2 vasoconstrictor responses observed in SHR compared to WKY. The effects of c-Src on vascular contraction seem to be explained not only by inhibition of TP activation, as demonstrated by the inhibition of U46619-induced contractile responses by PP1, but also by the inhibition of H_2O_2-induced TXA_2 production and suggest that c-Src is an early mediator of H_2O_2 effects.

We previously demonstrated that H_2O_2 contractile responses in SHR-but not in WKY- are dependent on $O_2^{\cdot -}$ derived presumably from NAD(P)H Oxidase activated by TXA_2 [6,7]. Herein, we extend these observations and demonstrated that the activation of NAD(P)H Oxidase activity and $O_2^{\cdot -}$ production induced by H_2O_2 in mesenteric arteries from SHR were dependent on TXA_2 and c-Src. Accordingly, TP activation increased NAD(P)H Oxidase activity in a c-Src dependent manner in vessels from hypertensive rats. Other studies have demonstrated that c-Src regulates vascular NAD(P)H Oxidase-derived $O_2^{\cdot -}$ generation after Angiotensin II [30,31], endothelin-1 [32] or H_2O_2 [30,33].

Role of ERK1/2

Other candidates to modulate H_2O_2 and TXA_2-induced contractile responses might be components of the MAPK family. ERK1/2 participates in the H_2O_2 contractile response in different vessels from normotensive animals [19,22]. In addition, Tabet et al [23] described that H_2O_2 induced ERK1/2 activation in cultured VSMC from SHR and WKY. More importantly, this activation was greater in cells from hypertensive than normotensive animals [23]. Inhibition of ERK1/2 with PD98059 inhibited H_2O_2 contractile responses only in SHR arteries. In addition, basal activation of ERK1/2 was

greater in MRA from to SHR than WKY. Interestingly, PD98059 did not modify U46619-induced contractions and the combination of SQ29548 plus PD98059 exerted greater inhibitory effects on H_2O_2 contraction than each inhibitor alone. These results suggest that ERK1/2 might participate in H_2O_2 contraction through TXA_2/TP independent pathways. In agreement with our study, inhibition of MAPK with PD98059 did not modify U46619-induced contraction in rat MRA from normotensive animals [17], although conflicting reports have been demonstrated depending on the vessel type and specie [17,34]. Supporting the role of ERK1/2 as the additional mediator involved in the hyperreactivity to H_2O_2 in SHR, U0126, another ERK1/2 inhibitor, blocked H_2O_2-induced NAD(P)H Oxidase activity and $O_2^{\cdot -}$ production in SHR, suggesting that the participation of ERK1/2 on H_2O_2 contraction might be due to NAD(P)H Oxidase-derived $O_2^{\cdot -}$. The role of ERK1/2 in NA(D)PH Oxidase activation has been described for other stimuli like Angiotensin II [30,35].

A novel aspect derived from this study is the role of c-Src, ERK1/2 and TXA_2 in the relationship triggered by H_2O_2 between mitochondria and NAD(P)H Oxidase. This is an important aspect since it has been demonstrated that this relationship is key to explain hypertension and endothelial dysfunction induced by Angiotensin II [12,13]. Our data demonstrate that H_2O_2-induced TXA_2 is an important mediator of the loss of mitochondrial membrane potential that could increase mitochondrial ROS production and further NAD(P)H Oxidase activation in SHR vessels. This is also supported by the fact that U46619 mimicked the effects of H_2O_2 on mitochondrial membrane potential and NAD(P)H Oxidase activation. Interestingly, the intracellular signalling pathways responsible of these effects are c-Src and ERK1/2 which as mentioned, are crucial to explain vascular effects of H_2O_2. More importantly, this has a role in vascular function

since H_2O_2 responses in SHR arteries were greatly diminished by selective mitoROS blockade.

Role of Rho Kinase

The RhoA protein activation by contractile agonists allows the stimulation of Rho Kinase which mainly promotes muscle contraction through phosphorylation of myosin light chain phosphatase, thus playing a key role in Ca^{2+} sensitization induced by agonists [36]. It is generally accepted that RhoA/Rho Kinase expression and/or activation is greater in the hypertensive vasculature and participates of hypertension development [20,37-39]. In agreement, we observed that Rho kinase expression was greater in MRA from SHR than WKY rats. Previous studies have demonstrated that ROS, particularly $O_2^{\cdot}$, can activate Rho Kinase and mediate vascular contraction [40,41]. Moreover, Chandra et al. [42] recently described that H_2O_2 can induce Rho Kinase translocation in endothelial cells. We observed that Y27632 inhibited H_2O_2 and U46619-induced contractile responses only in arteries from SHR, suggesting a role of Rho Kinase in vascular contraction in hypertension. In addition, these results suggest that the role of Rho Kinase in H_2O_2-induced responses in SHR might be related with the activation of TP receptors. In support of this hypothesis, no further inhibition was observed when Y27632 and SQ29548 were combined. Participation of Rho Kinase in H_2O_2-contractile response has been described [19-22] although conflicting results have been described depending on the vascular beds or the precontractile agent used [18,19,37,43]. Rho Kinase inhibitors did not modify U46619 contractions in normotensive mesenteric arteries [34]. In addition, Rho Kinase was not activated by H_2O_2 in pulmonary arteries [41] and it was not involved in H_2O_2 induced sustained contractile response in the same vascular bed from normotensive animals [44]. Interestingly, Dennis et al., [20] demonstrated that H_2O_2 elicits a greater contraction in

carotid artery from SHR than WKY that is TP-dependent and mediated by Rho Kinase in both strains. Besides its effects of VSM contraction, Rho Kinase plays a crucial role on the development of cardiovascular diseases through ROS production, inflammation, and VSMC proliferation, among others [45]. Rho Kinase upregulates different subunits of the NAD(P)H Oxidase (nox1, nox4, gp91phox, and p22phox) and increases Angiotensin II-induced ROS production [46]. However, in our experimental conditions, Rho Kinase was not involved in the activation of NAD(P)H Oxidase activity elicited by H_2O_2 in MRAs from SHR.

A final issue deserves further consideration. In addition of the different signaling pathways involved in H_2O_2 or TXA_2 responses, we should not forget that H_2O_2 induces greater TXA_2 production in SHR than in WKY. Therefore, it is highly possible that the hyperresponsiveness to exogenous H_2O_2 observed in the SHR mesenteric arteries might be also explained by the H_2O_2-derived TXA_2 overproduction that acting *via* TP receptors elicits activation of RhoA/Rho Kinase and c-Src.

Conclusions

Our results demonstrate that activation of signaling pathways such as ERK1/2, c-Src and Rho Kinase are key to explain the increased vasoconstrictor responses induced by H_2O_2 in vessels from hypertensive animals by modulating not only TXA_2 production and/or signaling but also the existing relationship between mitochondria and NADPH oxidase.

Perspectives

The contribution of ROS to vascular damage in hypertension might be explained not only by the increased ROS bioavailability observed systemically or locally in hypertensive patients [8], but also by the enhanced redox signaling developed in these

conditions [47]. Our data further build on this notion demonstrating that different intracellular pathways are involved in H_2O_2 vascular effects in normotensive and hypertensive animals. Moreover, we demonstrate that oxidative stress is an important perpetuator of key inflammatory pathways including prostanoids and mitochondrial oxidative stress which act in relationship to induce vascular dysfunction. Improving our knowledge of H_2O_2 signaling at vascular level particularly in pathological conditions, will help us to develop more specific or more directed strategies to regulate ROS effects to treat hypertensive vascular damage.

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Conflict of interest

None

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Figure legends

Figure 1. Effect of SQ29548 (1 µmol/L) and PP1 (1 µmol/L) on the concentration–response curves to H_2O_2 (A) and U46619 (B) in phenylephrine-contracted mesenteric resistance arteries from Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Results are expressed as a percentage of K^+-KHS contraction. ***P<0.001 *vs* control. (C) Effect of H_2O_2 in the absence and in the presence of PP1 on thromboxane B_2 production by mesenteric resistance arteries from WKY and SHR. *P<0.05 *vs* WKY, #P<0.05 *vs* Control, †P<0.05 vs H_2O_2. (D) Densitometric analysis and representative western blots for $pTyr^{418}$c-Src and c-Src protein expression in mesenteric resistance arteries from WKY and SHR. *P<0.05 *vs* WKY. Number of animals is indicated in parenthesis.

Figure 2. Effect of PD98059 (10 μmol/L), SQ29548 (1 μmol/L) or PD98059 plus SQ29548 on the concentration–response curves to H_2O_2 (A) or U46619 (B) in phenylephrine-contracted mesenteric resistance arteries from Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Results are expressed as a percentage of K^+-KHS contraction. *P<0.05, ***P<0.001 *vs* control; †P<0.05 *vs* PD98059 or SQ29548. (C) Densitometric analysis and representative western blots for pERK1/2 protein expression in mesenteric resistance arteries from WKY and SHR. GADPH was used as loading control. *P<0.05 *vs* WKY. Number of animals is indicated in parenthesis.

Figure 3. Effect of Y27632 (1 μmol/L) or Y27632 plus SQ29548 (1 μmol/L) on the concentration–response curves to H_2O_2 (A) or U46619 (B) in phenylephrine-contracted mesenteric resistance arteries from Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Results are expressed as a percentage of K^+-KHS contraction. *P<0.05; **P<0.01 ***P<0.001 *vs* control. C) Densitometric analysis and representative western blot for Rho kinase protein expression in mesenteric resistance arteries from WKY and SHR. GADPH was used as loading control. *P<0.05 *vs* WKY. Number of animals is indicated in parenthesis.

Figure 4. A) Effect of H_2O_2 (100 µmol/L) in the absence or in the presence of furegrelate (1 µmol/L), U0126 (10 µmol/L), PP1 (1 µmol/L) or Y26732 (1 µmol/L) on NAD(P)H Oxidase activity in mesenteric resistance arteries from spontaneously hypertensive (SHR) rats. B) Representative fluorescence confocal photomicrographs and quantitative analysis of vascular superoxide anion production in mesenteric resistance arteries from SHR untreated and treated with H_2O_2 in the absence and the presence of furegrelate, U0126 or PP1. Image size 375x375 µm. Images were captured with a fluorescence confocal microscope (x40 oil immersion objective, zoom 1). C) Effect of U46619 (10 µmol/L) in the absence or in the presence of PP1, on NAD(P)H Oxidase activity in aorta from SHR. Data are expressed as a percentage of control arteries. **P<0.01; ***P<0.001 *vs* Control; #P<0.05; ##P<0.01 *vs* H_2O_2 or U46619. Number of animals is indicated in parenthesis.

Figure 5. A) Effect of mito-TEMPO (1 μmol/L) on the concentration–response curve to H_2O_2 in phenylephrine-contracted mesenteric resistance arteries from spontaneously hypertensive (SHR) rats. Results are expressed as a percentage of K^+-KHS contraction. B) Effect of H_2O_2 (100 μmol/L) in the absence or in the presence of mito-TEMPO or rotenone (1 μmol/L), on NAD(P)H Oxidase activity in aorta from SHR rats. Data are expressed as a percentage of control arteries. C) Effect of H_2O_2, in the absence or in the presence of furegrelate (1 μmol/L), U0126 (10 μmol/L) or PP1 (1 μmol/L), on mitochondrial membrane potential in mesenteric resistance arteries from SHR rats. Data are expressed as a percentage of control arteries. D) Effect of U46619 (10 μmol/L), in the absence or in the presence of PP1, on mitochondrial membrane potential in aorta from SHR rats. Data are expressed as a percentage of control arteries. **P<0.01, ***P<0.001 *vs* control. #P<0.05, ##P<0.01 *vs* H_2O_2 or U46619. Number of animals is indicated in parenthesis.

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Figure 6. Representative diagram of the signaling pathways involved in the contractile response induced by H_2O_2 in mesenteric resistance arteries from Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. In WKY, H_2O_2 contractile response is mediated by c-Src dependent TXA_2 production that further activates c-Src. In arteries from SHR c-Src participates in H_2O_2-induced TXA_2 release which further activates Rho Kinase, c-Src and the relationship between mitochondria and NAD(P)H Oxidase to mediate contractile effects. In addition, TXA_2-independent activation of ERK1/2 by H_2O_2 contributes to H_2O_2 contraction in SHR trough effects on mitochondria and NAD(P)H Oxidase. All together, the greater TXA_2 and $O_2^{\cdot-}$ production and the different signaling pathways involved explain the increase in the contractile response induced by H_2O_2 in hypertension. Furegrelate is inhibitor of TXA_2 synthase, PP1 inhibits c-Src, PD98059 and U0126 are ERK1/2 MAPK inhibitors, SQ29548 is a TP antagonist, Y27632 inhibits Rho Kinase, mito-TEMPO and Rotenone inhibit mitochondrial ROS.

Figure 1

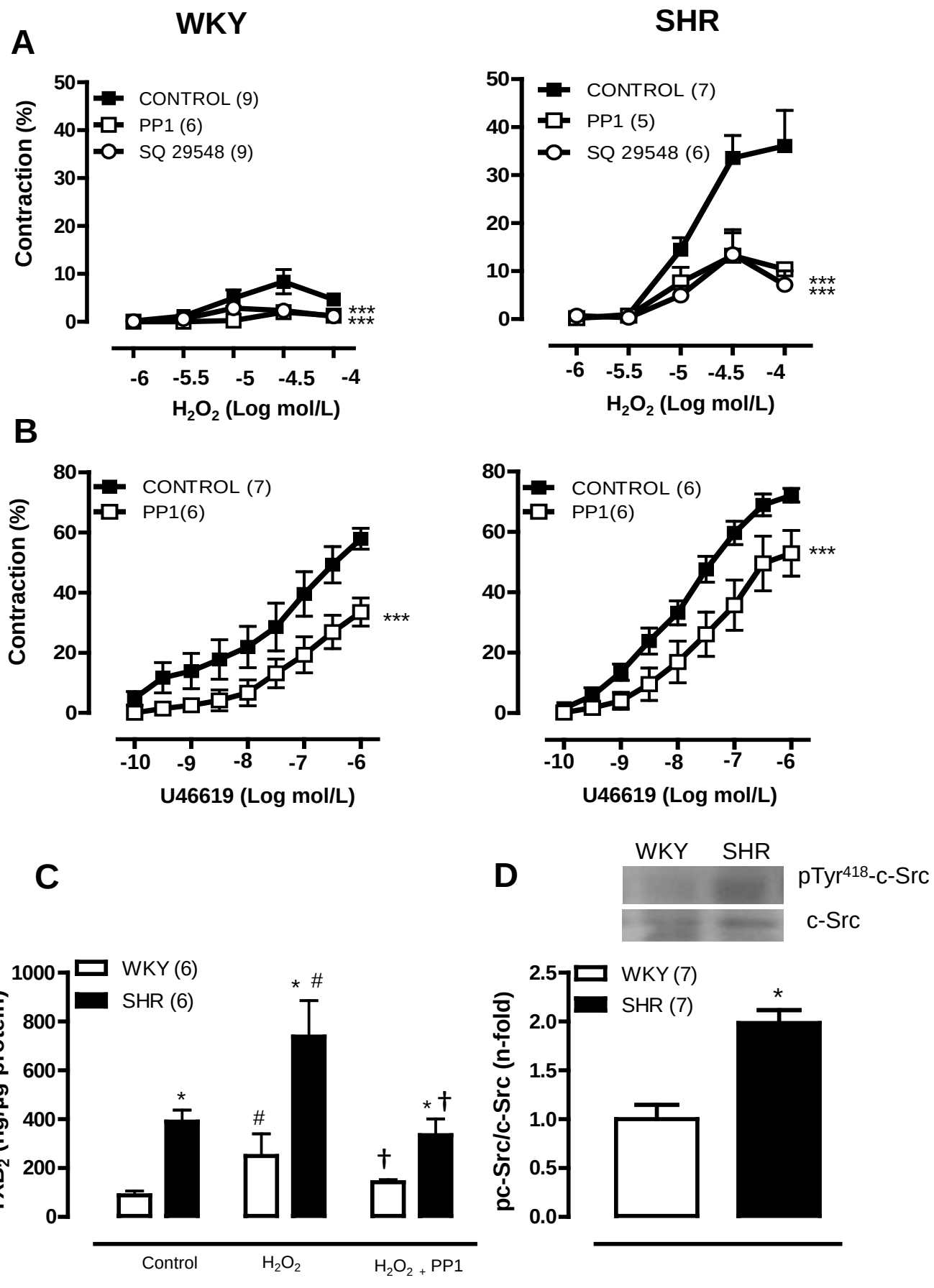
WKY
SHR
A
Contraction (%)
CONTROL (9)
PP1 (6)
SQ 29548 (9)
CONTROL (7)
PP1 (5)
SQ 29548 (6)
***
***
H2O2 (Log mol/L)
B
CONTROL (7)
PP1(6)
CONTROL (6)
PP1(6)
U46619 (Log mol/L)
C
TXB2 (ng/µg protein)
WKY (6)
SHR (6)
Control
H2O2
H2O2 + PP1
D
WKY
SHR
pTyr418-c-Src
c-Src
pc-Src/c-Src (n-fold)
WKY (7)
SHR (7)

Figure 2

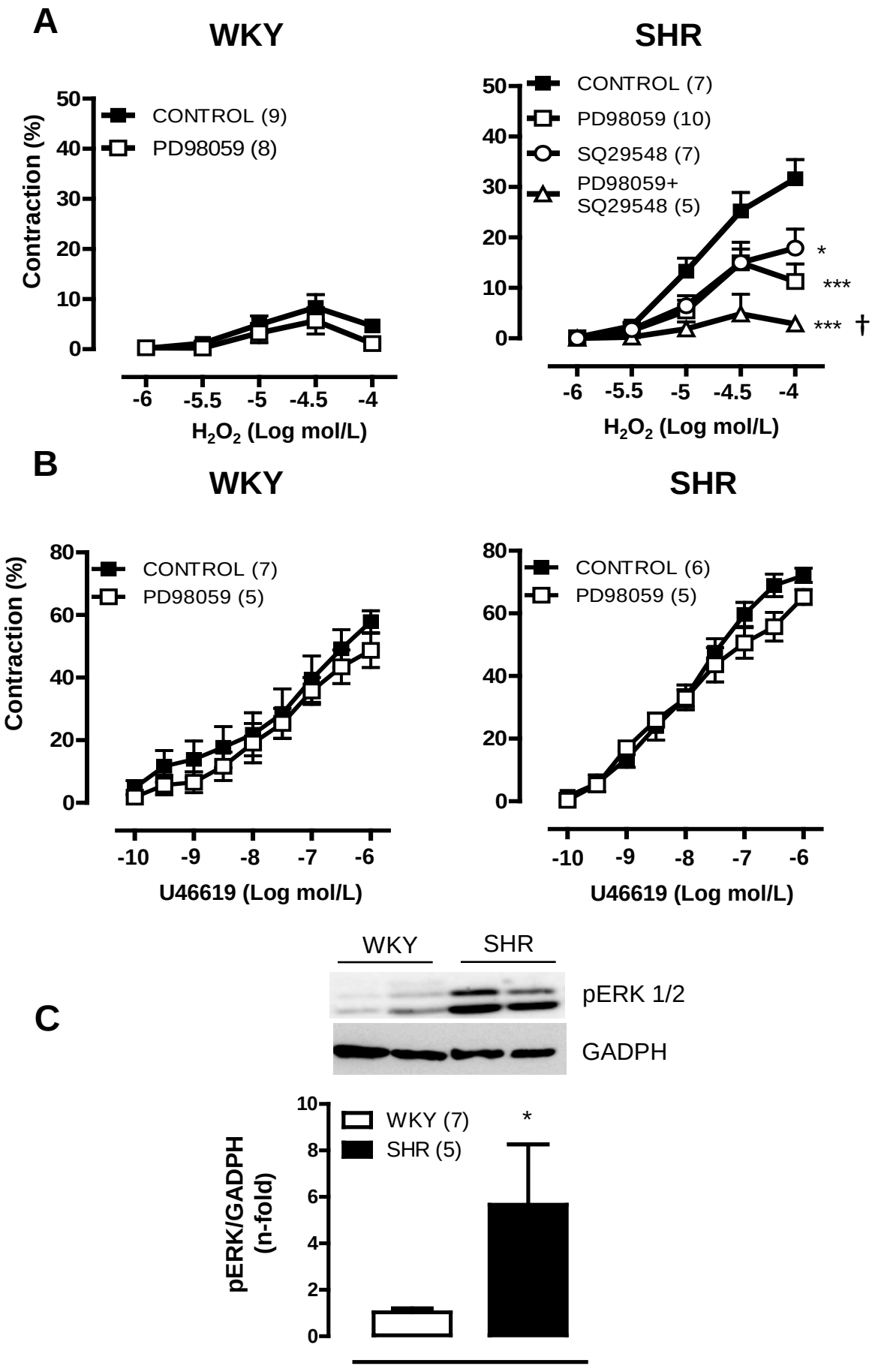
A
WKY
SHR
Contraction (%)
CONTROL (9)
PD98059 (8)
CONTROL (7)
PD98059 (10)
SQ29548 (7)
PD98059+ SQ29548 (5)
*
***
*** †
H2O2 (Log mol/L)
B
WKY
SHR
Contraction (%)
CONTROL (7)
PD98059 (5)
CONTROL (6)
PD98059 (5)
U46619 (Log mol/L)
C
WKY
SHR
pERK 1/2
GADPH
pERK/GADPH (n-fold)
WKY (7)
SHR (5)
*

Figure 3

Figure 3

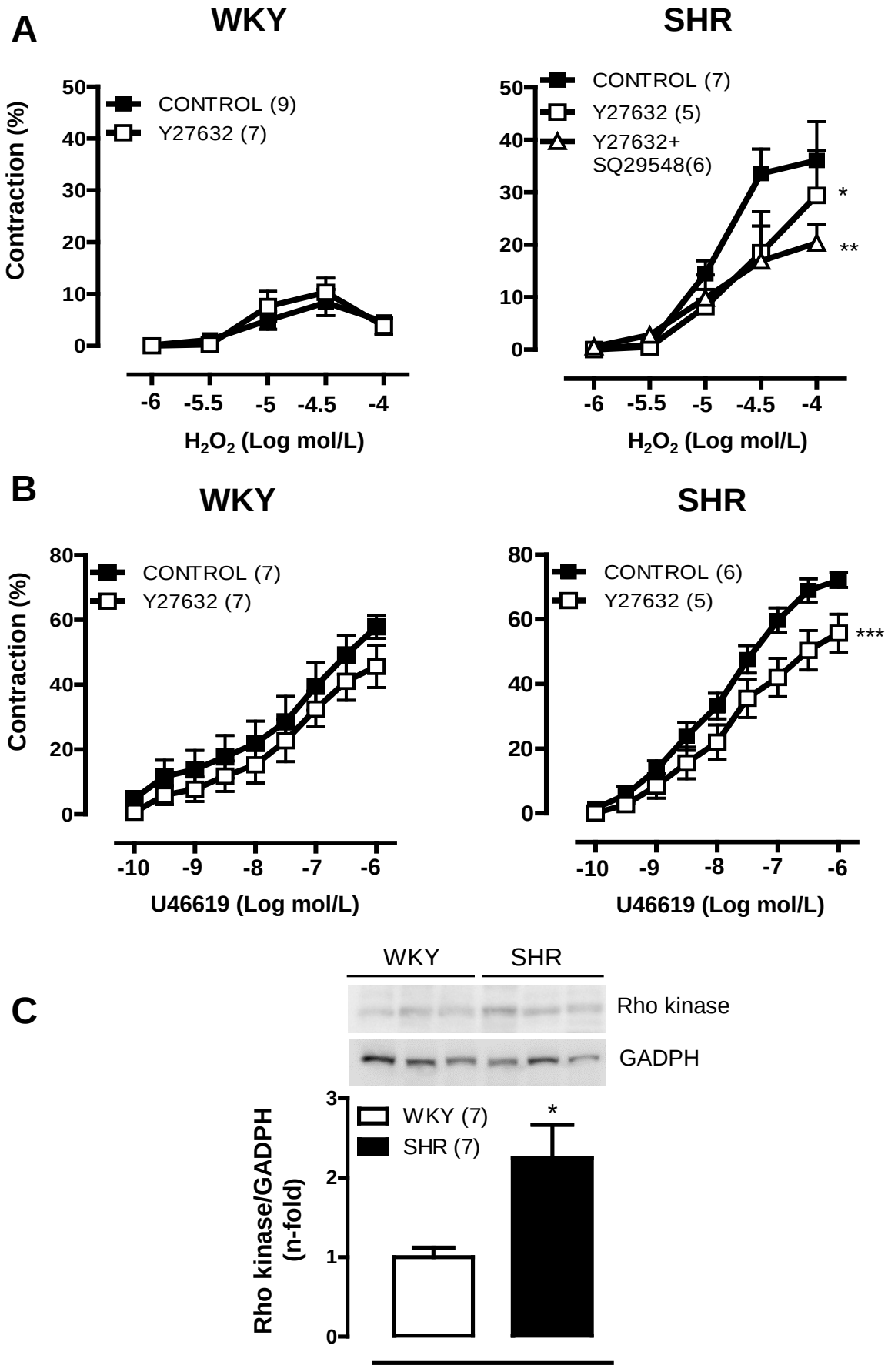

Figure 4

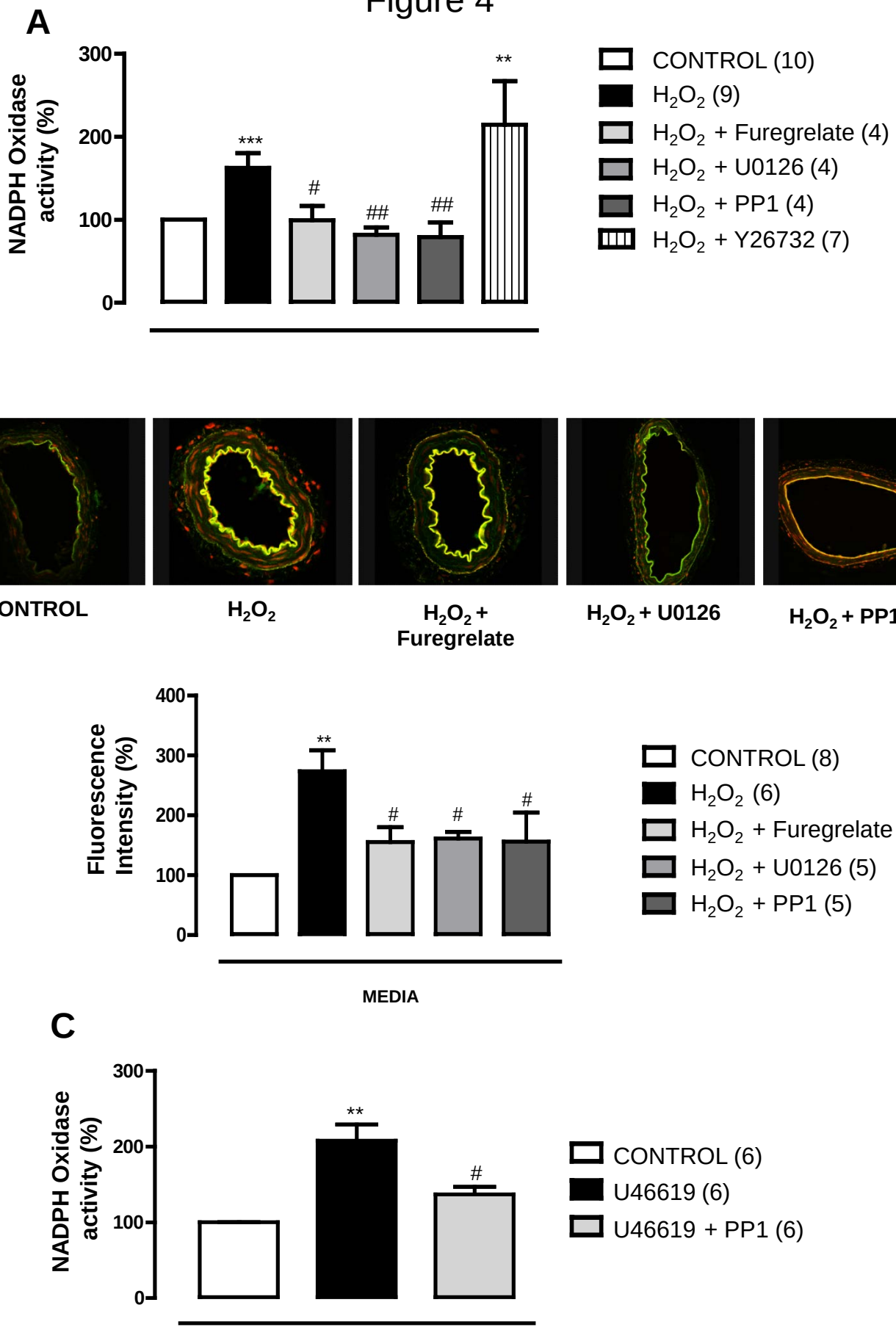
A
NADPH Oxidase activity (%)
300
200
100
0
***
#
##
##
**
CONTROL (10)
H2O2 (9)
H2O2 + Furegrelate (4)
H2O2 + U0126 (4)
H2O2 + PP1 (4)
H2O2 + Y26732 (7)
B
CONTROL
H2O2
H2O2 + Furegrelate
H2O2 + U0126
H2O2 + PP1
Fluorescence Intensity (%)
400
300
200
100
0
**
#
#
#
CONTROL (8)
H2O2 (6)
H2O2 + Furegrelate (8)
H2O2 + U0126 (5)
H2O2 + PP1 (5)
MEDIA
C
NADPH Oxidase activity (%)
300
200
100
0
**
#
CONTROL (6)
U46619 (6)
U46619 + PP1 (6)

Figure 5

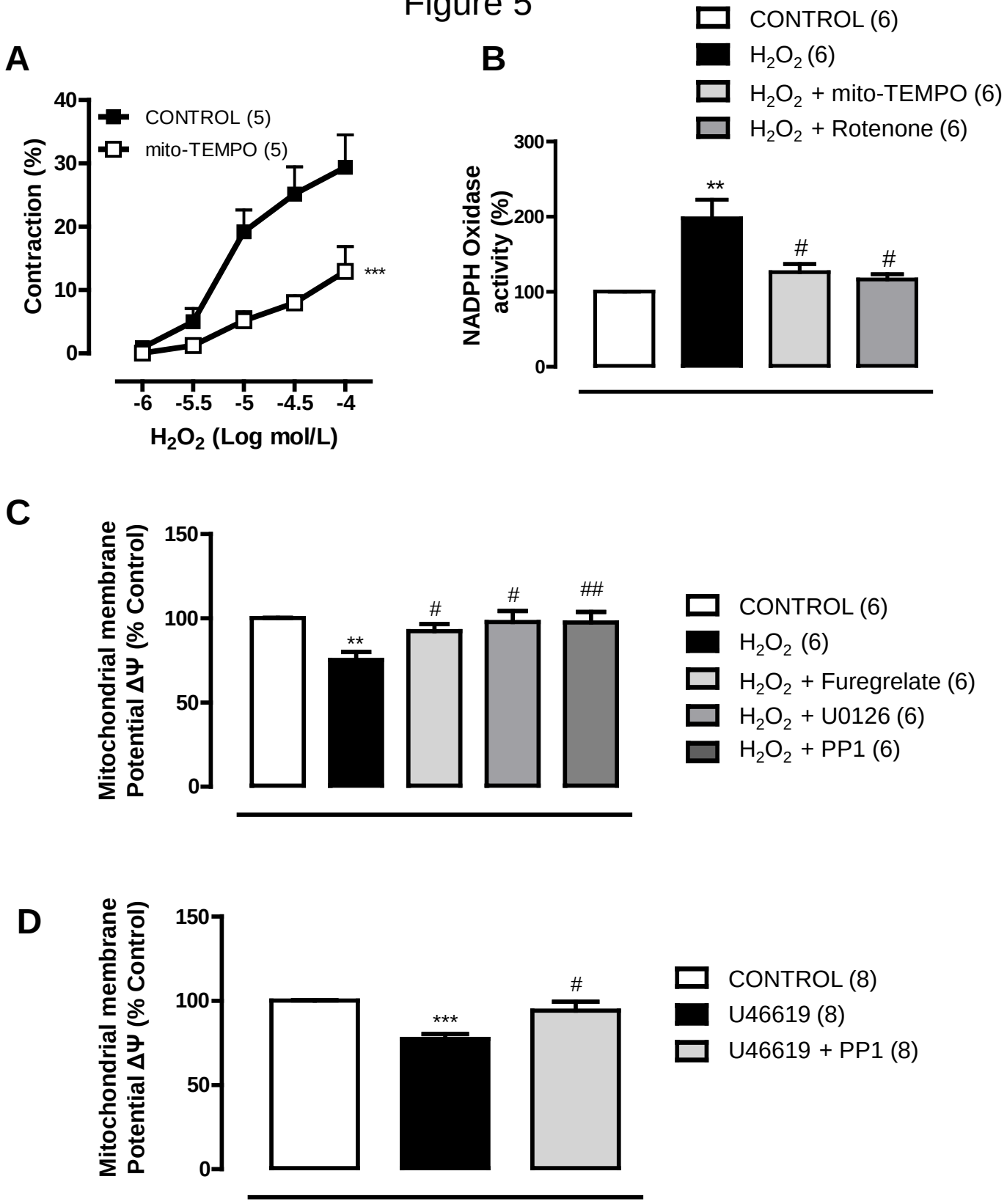
A
CONTROL (5)
mito-TEMPO (5)
Contraction (%)
40
30
20
10
0
***
-6
-5.5
-5
-4.5
-4
H2O2 (Log mol/L)
B
CONTROL (6)
H2O2 (6)
H2O2 + mito-TEMPO (6)
H2O2 + Rotenone (6)
NADPH Oxidase activity (%)
300
200
100
0
**
#
#
C
Mitochondrial membrane Potential ΔΨ (% Control)
150
100
50
0
**
#
#
##
CONTROL (6)
H2O2 (6)
H2O2 + Furegrelate (6)
H2O2 + U0126 (6)
H2O2 + PP1 (6)
D
Mitochondrial membrane Potential ΔΨ (% Control)
150
100
50
0
***
#
CONTROL (8)
U46619 (8)
U46619 + PP1 (8)

Figure 6 black and white

Figure 6

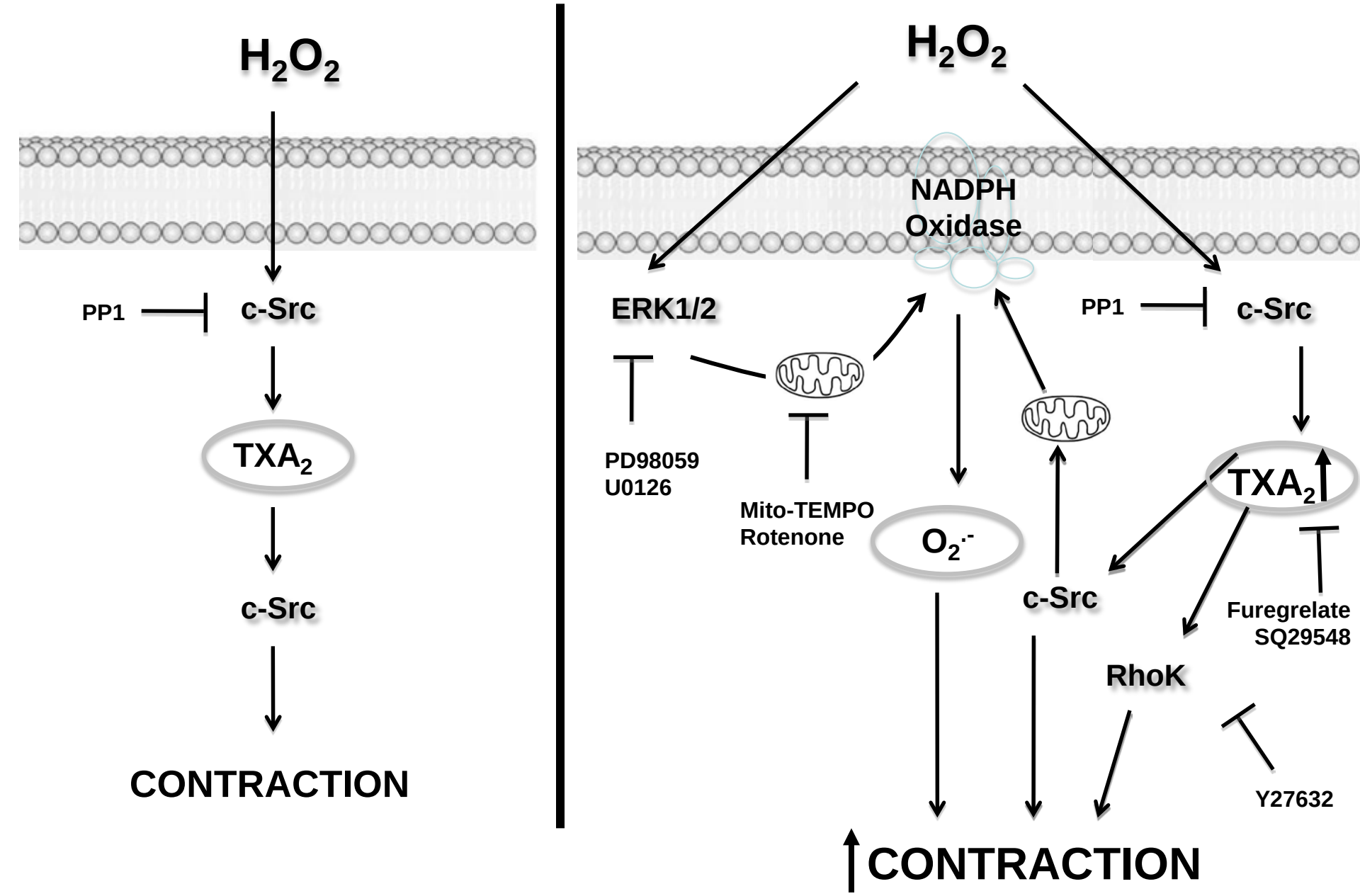

Figure 6 black and white

Figure 6