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Hydrogen peroxide acts as relaxing factor in human vascular smooth muscle cells independent of map-kinase and nitric oxide

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1. ABSTRACT

We previously showed that hydrogen peroxide (H_2O_2) induced resistance artery relaxation independent of endothelium. Thus, in this study we investigated the mechanism of relaxation induced by H_2O_2 on human renal vascular smooth muscle cell (HVSMC). HVSMC were stimulated with H_2O_2 and/or angiotensin II (Ang II), proline-rich-tyrosine-kinase-2 (PYK2), ERK1/2 MAP-Kinase, and myosin light chain 20 phosphorylation (Lc20) were assessed using Western blot analysis in the presence of potassium channel blockers, MAP-Kinase, and nitric oxide synthesis (NOS) inhibitors. H_2O_2 increased PYK2 and ERK1/2 phosphorylation, and at the same time decreased Lc20 phosphorylation. AngII increased phosphorylation of PYK2, ERK1/2 and Lc20, whereas, the pretreatment of HVSMC with H_2O_2 decreased Lc20 phosphorylation induced by AngII. MEK inhibition, decreased ERK1/2 phosphorylation, but had no effect on the inhibition of phosphorylation of Lc20 induced by H_2O_2. The inhibition of Ca^{2+}-dependent K^+ channels (BKCa) and NOS did not block the decrease of Lc20 phosphorylation in response to H_2O_2. On the other hand, pretreatment of HVSMC with 60 mM of KCl, increased rather than decreased Lc20 phosphorylation in response to H_2O_2. This study shows the evidence that H_2O_2 acts as a relaxing factor and as an activator of PYK2 and ERK1/2 in Human renal VSMC. The relaxation induced by H_2O_2 is independent of BKCa, ERK1/2 MAP-Kinase and NOS pathways. The relaxing effect to H_2O_2 changes to contracting effect when the potassium channels are compromised.

2. INTRODUCTION

Vascular smooth muscle cells (VSMC) play an important role in maintaining vascular homeostasis. To control artery tone, VSMC are subject to relaxation and contraction. Indeed, two well-known vasodilator factors: Nitric oxide and prostacyclin induce artery relaxation. In various blood vessels, endothelium-dependent relaxation can be accompanied by hyperpolarization of VSMC (1, 2). These endothelium-dependent relaxations and hyperpolarizations can be partially or totally resistant to inhibitors of nitric oxide synthesis and cyclooxygenases (3) and can occur without an increase in intracellular level of cyclic nucleotides in the VSMC. Therefore, the existence of an additional pathway that involved smooth muscle hyperpolarization/relaxation was suggested and attributed to a non-characterized endothelial factor called endothelium-derived hyperpolarization factor (EDHF) (4). The two pathways, that involved nitric oxide and prostacylin to induce artery relaxation, are well known while the nature and the mechanism of H_2O_2 as relaxing factor are not yet resolved.

Reactive oxygen species (ROS) are critically involved in many, *in vivo*, physiological, and pathological process. The mechanism that account for these *in vivo* processes have not been fully characterized, but a role for ROS has been implicated in signaling cascades and artery dysfunction. In addition, stretching is associated with changes in the vascular redox state. It has been shown that H_2O_2 may activate different signal transduction pathways that could regulate VSMC contractile function such as PYK2, ERK1/2 and p38 MAP kinases, and Akt (5-7). In contrast, H_2O_2 also acts as an endogenous EDHF released from endothelial cells *in vivo* and plays an important role in arterial autoregulation (8, 9). It has been demonstrated that H_2O_2 is a major dilatory factor released from the endothelium in mesenteric arteries isolated from endothelial nitric oxide synthesis knockout mice and humans (9, 10). However, it is unclear whether H_2O_2 consistently acts as a relaxing factor on human renal VSMC and the mechanism mediated-dilation is not yet clear. Different studies showed that H_2O_2 could induce VSMC hyperpolarization. Others studies demonstrate that H_2O_2-induced rabbit aorta vasodilatation mediated through nitric oxide release from the endothelium, whereas Thengchaisri and Kuo reported that H_2O_2 induces endothelium-dependent vasodilatation through COX-1-mediated release of PGE2 and also directly relaxes smooth muscle by hyperpolarization through KCa^{2+} channel activation (11). H_2O_2 response is still under deep debate and is not clear whether H_2O_2 is contributing to EDHF-mediated response (as an endothelial intracellular messenger) or is a diffusible factor from endothelial cells to VSMC. More complicated, it has been shown that neurohormones such Ang II and endothelin induced H_2O_2 release in conduct VSMC that may moderate the contraction magnitude and therefore control the contraction/relaxation balance induced by those factors (12-14).

We hypothesize that hydrogen peroxide can activate both intracellular signaling involved in artery contraction and relaxation. In this study, we used VSMC isolated from human renal artery stimulated with H_2O_2 to determine intracellular signaling cascades, involved in VSMC function.

3. MATERIALS AND METHODS

3.1. Materials

Anti-phosphorylated and total PYK2 and anti-phosphorylated Lc20 antibodies were purchased from Cell Signalling. Anti- phosphorylated and total ERK1/2 were purchased from Promega. AngII, Iberiotoxin, charybdotoxin, KCl, and L-NAME were purchase from Sigma. U0126 was purchased from Calbiochem.

3.2. Cell culture

Human vascular smooth muscle cell from renal artery were isolated by enzymatic digestion as described previously (15) HVSMC (*passages 3-10*) were maintained in culture at 37°C in smooth muscle growth medium fully supplemented with fetal bovine serum, growth factors, trace elements and antibiotics (Cell application, inc). HVSMC were grown to 75 ~ 80% confluence and then growth arrested for 48 h in serum-free DMEM supplemented with 0.1% BSA. After 48 h, the serum-free DMEM is changed for one-hour equilibration before experiments starting.

3.3. Experiment Protocol

Different experiments were performed in which the following were included after equilibration period and during the hydrogen peroxide stimulation period. The inhibitors were incubated for 20 minutes before stimulation with hydrogen peroxide: (1) no drugs, (2) Ang II (100 nmol/L), (3) Iberiotoxin/charybdotoxin (50 and 100 nmol/L respectively), (4) U0126 (MAP-Kinase inhibitor, 10 µM), (5) L-NAME (nitric oxide synthesis inhibitor, 100 µmol/L) and KCl (60 mM).

3.4. Western blot analysis

Cell lysates were prepared as described previously (15). Equal amounts of protein (25 µg) were resolved in 10% SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was performed using phosphorylation state-specific antibodies against ERK1/2 MAP kinases (1:5,000; Promega), PYK2 (pY881, 1:2,000; Cell signalling), or myosin light chain 20 (Lc20) (1:1,000; Cell signalling). Bands were visualized by enhanced chemiluminescence (ECL; Amersham) and quantified using NIH Image software.

3.5. Statistical analysis

Results are expressed as Mean±SEM. The effect of the different treatments with reference to control conditions was determined by ANOVA (with Bonferrroni post-hoc analysis). Probability values (p) <0.05 were considered as significant.

4. RESULTS

4.1. Effect of H_2O_2 on VSMC under basal conditions

Under basal conditions, application of hydrogen peroxide (H_2O_2) for 5 minutes on human vascular smooth muscle cell (HVSMC) significantly increased PYK2 and ERK1/2 phosphorylation in a dose dependent manner (10, 50 and 100 µM) (Figure 1A, B). At the same time, H_2O_2 significantly inhibited phosphorylation of myosin light chain 20 (Lc20, hallmark of VSMC relaxation/contraction) (Figure 1C).

4.2. Effect of H_2O_2 on VSMC under stimulated conditions with angiotensin II (Ang II)

The application of Ang II (100 nM) on HVSMC for 5 minutes significantly increased the PYK2, ERK1/2, and Lc20 phosphorylation (Figure 2). The pretreatment of HVSMC with H_2O_2 (50 µM) significantly increases the Phosphorylated PYK2 and ERK1/2 (Figure 2A, B) and in the same time, significantly decreased Lc20 phosphorylation induced by Ang II (Figure 2C).

4.3. Effect of potassium channel iberiotoxin/charybdotoxin sensitive

HVSMC were treated with a mixture of Iberiotoxin (50 nM, specific blocker of BKCa) and charybdotoxin (100 nM, blocks IKCa BKCa and some KV) for 20 minutes. Under these conditions, Ang II still increased PYK2, ERK1/2, and Lc20 phosphorylation (Figure 3). On the other hand, under the basal and stimulated condition by Ang II, the inhibition of potassium channel sensitive to Iberiotoxin/Charybdotoxin did not affect the inhibition of the Lc20 phosphorylation induced by H_2O_2 (Figure 3C). In addition, the treatment of VSMC with 60 mM of KCl increases Lc20 phosphorylation

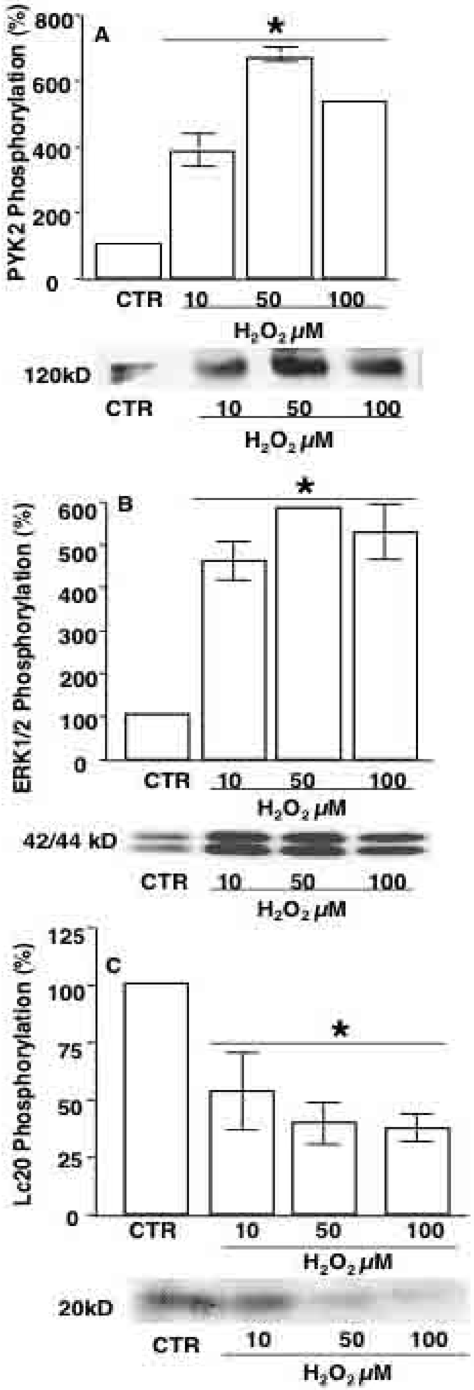

Figure 1. PYK2, ERK1/2 MAP-Kinase and Lc20 phosphorylation is induced by H_2O_2 in a time dependent manner. HVSMC incubated with serum-free DMEM for 48 hours, then treated with H_2O_2 dose-response manner (10-50-100 µM) for five minutes. Cell lysates were blotted with specific anti-phosphorylated PYK2 (A), ERK1/2 MAP-Kinase (B) and Lc20 (C). Quantified densitometry data were expressed as fold increases or decreases relative to control. Each experiment is a representative of 4 experiments. $P<0.05$ *statistically significant CTR vs. H_2O_2.

(Figure 4C). Under 60 mM of KCl, H_2O_2 potentiates rather than decreases Lc20 phosphorylation (Figure 4C).

4.4. Effect of MAP-Kinase and nitric oxide:

HVSMC treated with ERK1/2 inhibitor (U0126, 10 µM) significantly decreased the basal and stimulated ERK1/2 phsphorylation induced by Ang II (Figure 5B). The inhibition of ERK1/2 did not affect the decrease of Lc20 phosphorylation induced by H_2O_2 (Figure 5C). Similarly, the inhibition of nitric oxide synthesis (L-NAME, 100 µM) did not affect PYK2 and ERK1/2 phosphorylation increase, and inhibition of Lc20 phosphorylation in response to H_2O_2 under basal and stimulated conditions with Ang II (Figure 6).

5. DISCUSSION

Recently, we have found that exogenous hydrogen peroxide (H_2O_2) induced resistance artery relaxation endothelium-independent preconstricted with either pressure or phenylephrine (16). In this study we show the evidence that H_2O_2 acts as relaxing factor assessed by Lc20 phosphorylation decrease (hallmark of relaxation) in human renal VSMC. In addition, H_2O_2 also increases proline-rich tyrosine kinase 2 (PYK2) and MAP-Kinase (ERK1/2) phosphorylation involved in different cell functions such as contraction and structural remodeling. On the other hand, when potassium channels are compromised, rather to induce a relaxation, H_2O_2 increases Lc20 phosphorylation (hallmark of contraction).

Recently, properties and physiological functions of EDHF in vascular tissue were reviewed (17, 18). Thus, vascular tone relaxation nitric oxide and prostacylin dependent manner are largely documented. However, the nature and the signaling pathways of other relaxing factor (EDHF) are still poorly understood, especially on HVSMC. In this regard, our study focused on characterization and signaling pathways activated with H_2O_2 stimulation.

Reactive oxygen species (ROS) are implicated in patho-physiology of cardiovascular system. Thus, recent evidence suggests that certain forms of ROS such H_20_2 may act as signal transduction messengers in response to different agonists (19, 12). Indeed, H_20_2 could be a relaxing factor (EDHF) since it is released by endothelial and VSMC under special conditions and induces a relaxation of VSMC (20). Why have so many vastly different hypotheses been proposed concerning the identification of EDHF-mediated response? It has been proposed that EDHF could be low concentration of potassium (21), epoxyeicosatrienoic acids (22-25) or cAMP (22, 18). The possible explanation might suggest the presence of different EDHF isoforms and the potent effect of each isoform is dependent of the vessel bed.

In the present study, the application of exogenous H_2O_2 on HVSMC induced an increase of protein kinase redox sensitive such PYK2 and ERK1/2 MAP-Kinase phosphorylation. In addition, H_2O_2 decreased myosin light chain 20 phosphorylation (Lc20, hallmark of VSMC

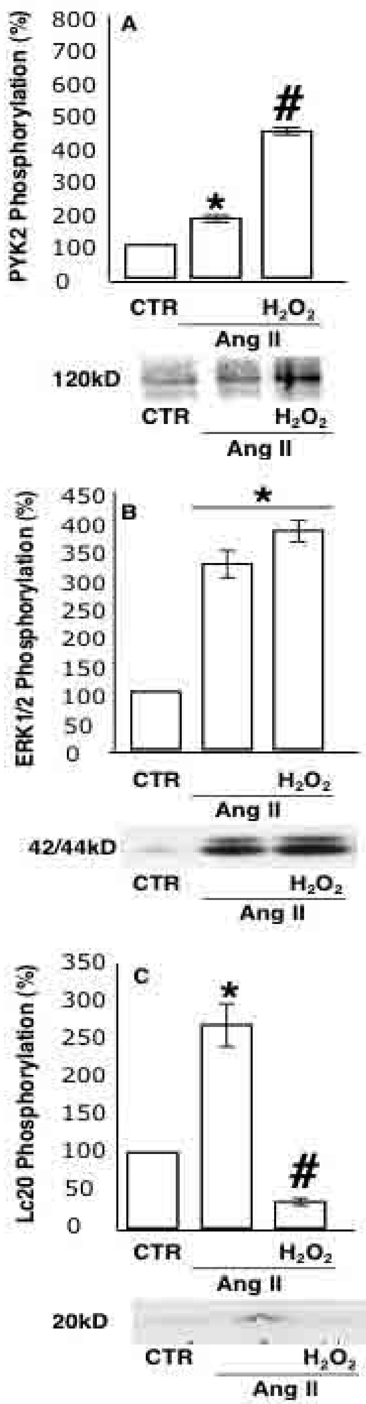

Figure 2. PYK2, ERK1/2 MAP-Kinase and Lc20 phosphorylation is induced by H_2O_2 (50 nM) and angiotensin II (100 nM). HVSMC incubated with serum-free DMEM for 48 hours, then treated with H_2O_2 (50 µM) and angiotensin II (100 nM) for five minutes. Cell lysates were blotted with specific anti-phosphorylated PYK2 (A), ERK1/2 MAP-Kinase (B) and Lc20 (C). Quantified densitometry data expressed as fold increases or decreases relative to control. Each experiment is a representative of 4 experiments. $P<0.05$ *statistically significant CTR vs. H_2O_2.

relaxation). This result indicates that H_2O_2 acts not only as relaxing factor but also as PYK2 and ERK1/2 MAP-Kinase activator that are important in mediating cellular contraction, growth, survival and migration. Supporting our data concerning the activation of PYK2, Frank *et al.*, have shown that reactive oxygen species in VSMC activate the non-receptor tyrosine kinase (26). In this study we showed the relaxing effect of H_20_2 assessed by Lc20 phosphorylation decrease in HVSMC and in agreement with recent studies showing that H_20_2 mediated vasodilation of canine arterioles and mouse resistance artery (20).

It has been recently shown that H_2O_2 is an EDHF (9, 10). To test this observation in our model, and to understand if H_2O_2 is an EDRF/EDHF or may induce EDRF/EDHF release, we treated HVSMC using the mixture of potassium channel blockers (Charybdotoxin and iberiotoxin), which blocked the intermediate and large conductance potassium channel ($BKCa^{2+}$) (27). Thus, the treatment of HVSMC with charybdotoxin and iberiotoxin decreased PYK2 and MAP-Kinase phosphorylation but did not block Lc20 phosphorylation decrease. Our data suggest that relaxation, assessed with Lc20 dephosphorylation, induced by H_2O_2 might involve different pathways others than potassium channel charybdotoxin and iberiotoxin sensitive. It is possible that the relaxation induced by H_2O_2 could involve others potassium channels. Surprisingly, upon 60 mM of potassium, H_2O_2 induced, rather than Lc20 phosphorylation decrease, Lc20 phosphorylation potentiation indicating an increase of contraction. These data are supported by our recent study showing that H_2O_2 increase resistance artery contraction under potassium stimulation (16).

Supporting our results, it has been shown that $ONOO^-$-triggered relaxation of canine cerebral arteries mediated by elevation of cyclic guanosine monophosphate (cGMP) levels, membrane hyperpolarization via K^+ channel activation, increase myosin light chain phosphatase activity, and interference with calcium movement and cellular membrane Ca^{2+} entry (28). The major problem is that we do not have the real evidence about the link between membrane potential and Lc20 phosphorylation in the same preparation. Therefore, it is very difficult to link between hyperpolarisation and Lc20 phopsphorylation decrease under H_2O_2 related to technical problematic between patch-clamp and cell culture signaling.

As mentioned above and determined by different studies, H_2O_2 can activate different signaling pathways (29). Previously others and we have shown that H_2O_2 activates kinase redox sensitive MAP-Kinase (29). To investigate the possible role of MAP-kinase that might modulate the effect of H_20_2 on Lc20 posphorylation, we treated HVSMC with MAP-Kinase inhibitor (U0126) followed by stimulation with H_2O_2. The inhibition of ERK1/2 MAP-Kinase significantly decreased ERK1/2 phosphorylation under basal and stimulated cell with Ang II. However, under these conditions, Lc20 phosphorylation decrease induced by H_20_2 was not prevented. These results show that ERK1/2 MAP-Kinase, even activated by H_20_2, is not

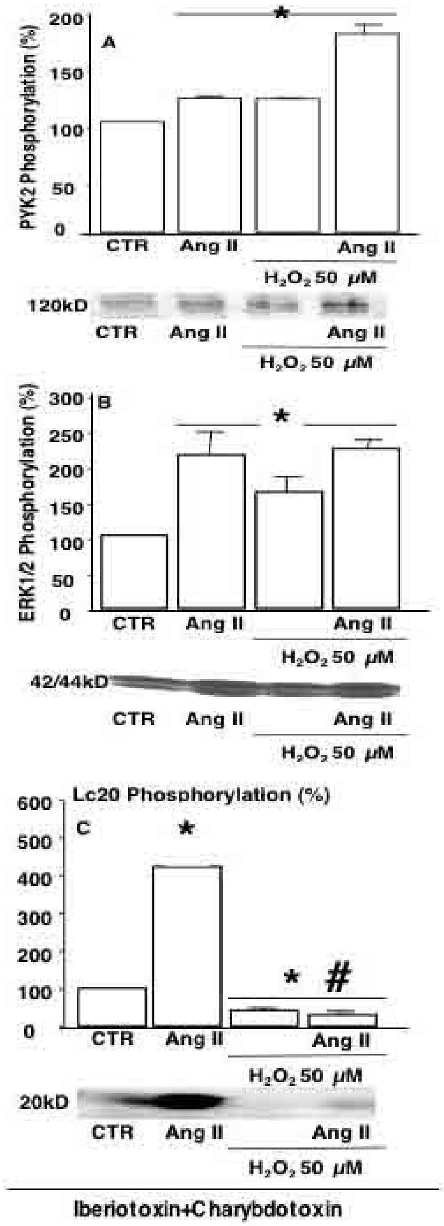

Figure 3. PYK2, ERK1/2 MAP-Kinase and Lc20 phosphorylation is induced by H_2O_2 (50 nM) and angiotensin II (100 nM) under iberiotoxin (100 nM) and charybdotoxin (50 nM) treatment. HVSMC incubated with serum-free DMEM for 48 hours, then treated with H_2O_2 (50 µM) and angiotensin II (100 nM) for five minutes. Cell lysates were blotted with specific anti-phosphorylated PYK2 (A), ERK1/2 MAP-Kinase (B) and Lc20 (C). Quantified densitometry data expressed as fold increases or decreases relative to control. Each experiment is a representative of 4 experiments. $P<0.05$ *statistically significant CTR vs. H_2O_2; # statistically significant Angiotensin II vs. angiotensin II + H_2O_2

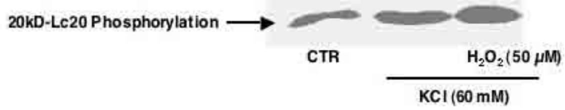

Figure 4. Lc20 phosphorylation induced by 60 mM of KCl and H_2O_2 (50 nM). HVSMC incubated with serum-free DMEM for 48 hours, then treated with KCl (60 mM) without and with H_2O_2 (50 µM). Cell lysates were blotted with specific anti-phosphorylated Lc20 (C). Quantified densitometry data expressed as fold increases or decreases relative to control. Each experiment is a representative of 4 experiments.

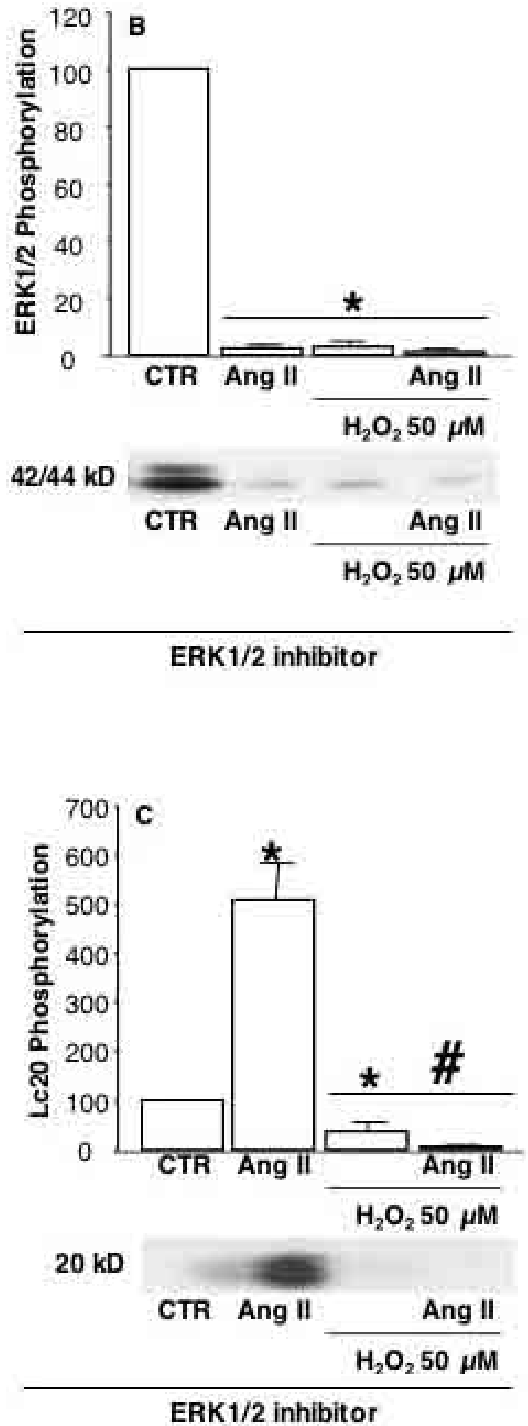

Figure 5. ERK1/2 MAP-Kinase and Lc20 phosphorylation is induced by H_2O_2 (50 nM) and angiotensin II (100 nM) under ERK1/2 MAP-Kinase inhibition (U0126, 10 µM). HVSMC incubated with serum-free DMEM for 48 hours, then treated with H_2O_2 (50 µM) and angiotensin II (100 nM) for five minutes. Cell lysates were blotted with specific anti-phosphorylated ERK1/2 MAP-Kinase (A) and Lc20 (B). Quantified densitometry data expressed as fold increases or decreases relative to control. Each experiment is a representative of 4 experiments. $P<0.05$ *statistically significant CTR vs. H_2O_2; # statistically significant Angiotensin II vs. angiotensin II + H_2O_2

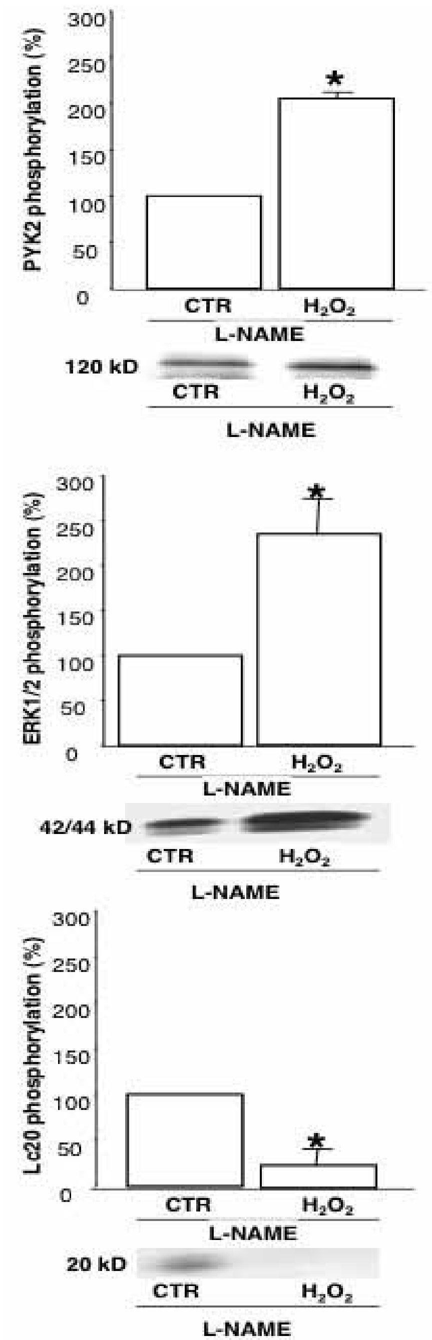

Figure 6. PYK2, ERK1/2 MAP-Kinase and Lc20 phosphorylation is induced by H_2O_2 (50 nM) and angiotensin II (100 nM) under nitric oxide synthesis inhibition (L-NAME, 100 µM). HVSMC incubated with serum-free DMEM for 48 hours, then treated with H_2O_2 (50 µM) and angiotensin II (100 nM) for five minutes. Cell lysates were blotted with specific anti-phosphorylated PYK2 (A), ERK1/2 MAP-Kinase (B) and Lc20 (C). Quantified densitometry data expressed as fold increases or decreases relative to control. Each experiment is a representative of 4 experiments. $P<0.05$ *statistically significant CTR vs. H_2O_2.

involved in signaling cascade of H_2O_2 to decrease Lc20 phosphorylation. The activation of PYK2 and ERK1/2 MAP-Kinase in response to H_2O_2 may play a different role than acting on Lc20 phosphorylation regulation. Thus, PYK2 and ERK1/2 MAP Kinase could be involved in sustained stimulation with H_2O_2 and may play role in gene regulation. Further experiments are needed to understand the role of PYK2 and ERK1/2 MAP-Kinase activation induced by H_2O_2.

It is well known that nitric oxide (NO) produced by endothelial NO synthase (NOS) diffuses to the underlying VSMC and modulates vascular tone as well as VSMC proliferation by increasing cGMP formation and protein kinase cGMP-dependent. Using supersensitive immunocytochemical technique to amplify signal with tyramide and electron microscopic immunogold labeling complemented with western blot analysis, it has been recently shown that the three nitric oxide synthesis (NOS) isoforms are present in VSMC (30). These NOS might play a role in modulating vascular tone under contraction induced by vasoconstrictor such Ang II. To confirm that Lc20 dephosphorylation induced by H_2O_2 may or may not involve the induction or neuronal NOS stimulation in HVSMC; we treated VSMC with L-NAME (NOS isoforms inhibitor). L-NAME did not prevent Lc20 phosphorylation decrease induced by H_2O_2 suggesting that NOS isoforms are not involved in Lc20 dephosphorylation. These results are in agreement with study showing that H_2O_2 may compensate coronary blood flow in the endocardium and in myocardial ischemia during coronary autoregulation (20).

The physiological significance of H_2O_2 as hyperpolarizing/relaxing factor is very important and might have therapeutic implications in cardiovascular disease such hypertension. In fact, in most cardiovascular diseases such hypertension and diabetes, artery relaxation involving nitric oxide is impaired (31, 32). Under these conditions, H_2O_2 might compensate a part of deleterious nitric oxide induced relaxation.

In conclusion, our study shows evidence of H_2O_2 as relaxing factor independently of nitric oxide, redox sensitive ERK1/2 MAP-Kinase and $BKCa^{2+}$. In addition, H_2O_2 acts also as an activator of different signaling pathways such as PYK2 and ERK1/2 MAP-Kinase that are important in cell contraction, growth, proliferation, and protein synthesis. Moreover, H_2O_2 can switch from relaxing to contracting factor when potassium channels are compromised.

6. ACKNOWLEDGEMENTS

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