

# Hypertension

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## **Losartan Metabolite EXP3179 Blocks NADPH Oxidase-Mediated Superoxide Production by Inhibiting Protein Kinase C: Potential Clinical Implications in Hypertension**

Ana Fortuño, Julen Bidegain, Pablo A. Robador, José Hermida, Jacinto López-Sagaseta, Oscar Beloqui, Javier Díez and Guillermo Zalba

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# Losartan Metabolite EXP3179 Blocks NADPH Oxidase-Mediated Superoxide Production by Inhibiting Protein Kinase C

## Potential Clinical Implications in Hypertension

Ana Fortuño, Julen Bidegain, Pablo A. Robador, José Hermida, Jacinto López-Sagasetta, Oscar Beloqui, Javier Díez, Guillermo Zalba

**Abstract**—Oxidative stress plays a critical role in the pathogenesis of hypertension. The NADPH oxidase constitutes a major source of superoxide anion in phagocytic cells, and its activation is associated with matrix metalloproteinase (MMP)-9 secretion by these cells. We investigated the effects of the angiotensin II type 1 receptor antagonist losartan and its metabolites (EXP3174 and EXP3179) on NADPH oxidase activity and MMP-9 secretion in human phagocytic cells. EXP3179, but not losartan and EXP3174, dose-dependently inhibited ( $P<0.05$ ) phorbol myristate acetate and insulin-stimulated NADPH oxidase activity. EXP3179 also inhibited phorbol myristate acetate-induced NADPH oxidase in endothelial cells. In addition, EXP3179 inhibited ( $P<0.05$ ) both phorbol myristate acetate-stimulated p47phox translocation from cytosol to membranes and protein kinase C activity. Affinity experiments and enzymatic assays confirmed that EXP3179 inhibited several protein kinase C isoforms. EXP3179 also inhibited ( $P<0.05$ ) phorbol myristate acetate-stimulated MMP-9 secretion. In a study performed in 153 hypertensive patients, phagocytic NADPH oxidase activity was lower ( $P<0.05$ ) in losartan-treated compared with untreated patients and in patients treated with other angiotensin II type 1 receptor antagonists or with angiotensin-converting enzyme inhibitors. Plasma levels of MMP-9 were lower ( $P<0.05$ ) in losartan-treated hypertensives compared with the other group of patients. Thus, EXP3179 acts as a blocker of the NADPH oxidase in phagocytic cells by a potential mechanism that targets the protein kinase C signaling pathway. This effect can be involved in reduced MMP-9 secretion by these cells. It is proposed that the EXP3179 metabolite may confer to losartan the specific capacity to reduce oxidative stress mediated by phagocytic cells in hypertensive patients. (*Hypertension*. 2009;54:744-750.)

**Key Words:** EXP3179 ■ hypertension ■ losartan ■ metalloproteinases ■ NADPH oxidase ■ PKC

Oxidative stress induced by reactive oxygen species, including superoxide anion ( $O_2^{\cdot-}$ ), is increased in arterial hypertension.<sup>1,2</sup> The NADPH oxidase systems constitute a major source of  $O_2^{\cdot-}$  in the vessel wall, and they are present in endothelial cells, smooth muscle cells, fibroblasts, and infiltrated monocytes/macrophages.<sup>3,4</sup> Protein kinase C (PKC)-dependent p47phox phosphorylation and translocation are major mechanisms involved in phagocytic NADPH oxidase activation.<sup>3</sup> Phagocytic NADPH oxidase is overactivated in essential hypertension.<sup>5,6</sup> Clinical studies have demonstrated that treatment with losartan improves endothelial function and decreases blood pressure through a reduction in oxidative stress.<sup>7,8</sup> Moreover, several experimental studies have demonstrated beneficial effects of losartan treatment on vascular alterations by blocking NADPH oxidase activation.<sup>9-12</sup>

Losartan is hepatically metabolized by the cytochrome-P450 pathway and exerts its antihypertensive actions predom-

inantly by EXP3174, its main metabolite and the pharmacological blocker of the angiotensin II type 1 receptor ( $AT_1R$ ).<sup>13,14</sup> Interestingly, during losartan metabolism, the liver also produces the EXP3179 metabolite, which has no  $AT_1R$ -blocking properties.<sup>14,15</sup> In recent years, several studies have reported that the EXP3179 metabolite exerts  $AT_1R$ -independent actions.<sup>15-18</sup> Krämer et al<sup>15</sup> demonstrated that EXP3179 mediates the anti-inflammatory properties of losartan by abolishing cyclooxygenase-2 upregulation. Furthermore, EXP3179 stimulates endothelial NO synthase and suppresses tumor necrosis factor- $\alpha$ -induced apoptosis.<sup>16</sup> Schupp et al<sup>17</sup> have demonstrated that EXP3179 acts as a peroxisome proliferator-activated receptor- $\gamma$  agonist. Finally, the ability of EXP3179 to inhibit collagen-dependent platelet activation has also been described.<sup>18</sup>

Therefore, we hypothesized that EXP3179 metabolite might inhibit NADPH oxidase in phagocytic cells by  $AT_1R$ -

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independent mechanisms. To test this hypothesis, we analyzed the ability of losartan and its metabolites, as well as other drugs interfering with the renin-angiotensin system, to block NADPH oxidase-dependent  $O_2^{\cdot-}$  production in human phagocytic cells. Given that matrix metalloproteinase (MMP)-9 secretion by phagocytic cells is regulated by NADPH oxidase,<sup>19</sup> we also assessed the effect of EXP3179 on this proteolytic enzyme. In addition, we evaluated NADPH oxidase-dependent  $O_2^{\cdot-}$  production and MMP-9 levels in phagocytic cells and plasma, respectively, obtained from hypertensive patients treated with different classes of drugs interfering with the renin-angiotensin system, including losartan.

## Methods

### Subjects

The study was performed in 2 different populations. The first population consisted of 50 apparently healthy subjects in whom the effect of different drugs that interfere with the renin-angiotensin system was analyzed on NADPH oxidase-dependent  $O_2^{\cdot-}$  production from phagocytic cells. In addition, the effect of EXP3179 was analyzed on phorbol myristate acetate (PMA)-induced p47phox translocation, PKC activity, and MMP-9 secretion. The second population consisted of 153 patients with the diagnosis of essential hypertension. These patients were considered hypertensive if they had sitting systolic and/or diastolic blood pressures of >139 and 89 mm Hg, respectively, or if they were taking antihypertensive medication during  $\geq 6$  months before the study. Blood pressure was measured on 3 occasions using a mercury sphygmomanometer, and the mean of these 3 readings was recorded. In this study, NADPH oxidase-dependent  $O_2^{\cdot-}$  production by phagocytic cells and plasma MMP-9 levels were evaluated in the patients classified in 4 groups: untreated patients, patients treated with losartan, patients treated with other AT<sub>1</sub>R antagonists (ARAs) different from losartan, and patients treated with angiotensin-converting enzyme inhibitors (ACEIs).

Both populations consisted of subjects who were referred to our institution for global cardiovascular risk assessment. Following institutional guidelines, the subjects were aware of the research nature of the study, and written informed consent was obtained from all of them. The study was performed in accordance with the Declaration of Helsinki, and the local committee on human research approved of the study protocol.

### Determination of NADPH Oxidase-Dependent $O_2^{\cdot-}$ Production

Peripheral blood mononuclear cells (PBMCs) were isolated with Lymphoprep from blood samples. The methodology used to isolate mononuclear cells yielded preparations with a very high purity ( $\geq 99\%$  were lymphocytes and monocytes). NADPH oxidase-dependent  $O_2^{\cdot-}$  production was measured by using 5  $\mu\text{mol/L}$  of lucigenin by a chemiluminescent method. Luminescence was measured in 400 000 cells every 15 to 30 s along an interval of 1 hour in a plate reader luminometer (Luminoskan Ascent, Labsystem). A buffer blank was subtracted from each reading, and the value of the area under the curve was used to quantify chemiluminescence. Data are expressed as relative light units produced per second. Although lucigenin concentration was low enough to avoid autoxidation, the measurements were validated against an independent measurement of  $O_2^{\cdot-}$  production using  $O_2^{\cdot-}$  dismutase-inhibitable ferricytochrome C reduction, as reported previously.<sup>6</sup> Previous experiments performed by our group allow us to confirm that NADPH oxidase is the main source of  $O_2^{\cdot-}$  production in response to PMA stimulation in phagocytic cells.<sup>6,19</sup>

Phagocytic NADPH oxidase-dependent  $O_2^{\cdot-}$  production in response to 3.2  $\mu\text{mol/L}$  of PMA in the presence (20 minutes of preincubation) of different doses of losartan, EXP3174, and EXP3179 was evaluated. In some experiments, the effects of

irbesartan, another ARA, and the effect of quinapril, an ACEI, were also evaluated. In addition, in some experiments, the effect of 100 nmol/L of human recombinant insulin on NADPH oxidase-dependent  $O_2^{\cdot-}$  production was evaluated in the presence (20 minutes of preincubation) of different doses of losartan, EXP3174, and EXP3179. Within the study performed in the hypertensive population, PMA-stimulated  $O_2^{\cdot-}$  production was also determined in PBMCs, following the same chemiluminescent methodology.

Finally, and to test whether EXP3179 could act as an  $O_2^{\cdot-}$  scavenger, we determined  $O_2^{\cdot-}$  production in a homogenate of protein from the rat kidney in the absence and presence of EXP3179. Briefly, we determined the effect of 100  $\mu\text{mol/L}$  of EXP3179 on  $O_2^{\cdot-}$  production from 100  $\mu\text{g}$  of renal homogenate in the presence of 5  $\mu\text{mol/L}$  of lucigenin during 5 minutes. In these experiments, we used as a positive control for the scavenging of  $O_2^{\cdot-}$ , 100 U/mL of  $O_2^{\cdot-}$  dismutase.

An expanded Methods section is available in the online Data Supplement at <http://hyper.ahajournals.org>.

## Results

### EXP3179 Metabolite Blocks $O_2^{\cdot-}$ Production

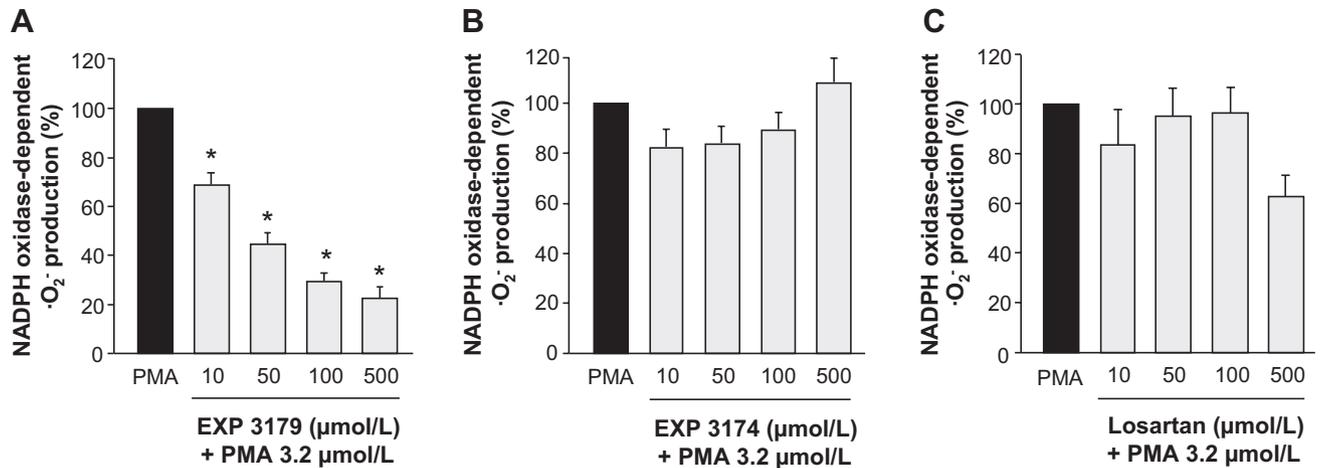
EXP3179 dose-dependently inhibited PMA-stimulated NADPH oxidase-mediated  $O_2^{\cdot-}$  production, reaching a significant effect at a concentration of 10  $\mu\text{mol/L}$  (Figure 1). The EC<sub>50</sub> of this metabolite for PMA-stimulated NADPH oxidase-mediated  $O_2^{\cdot-}$  production was 30  $\mu\text{mol/L}$ . In contrast, neither losartan nor EXP3174 influenced PMA-stimulated NADPH oxidase-mediated  $O_2^{\cdot-}$  production (Figure 1). Similarly, neither quinapril nor irbesartan influenced PMA-stimulated NADPH oxidase-dependent  $O_2^{\cdot-}$  production (Figure 2).

EXP3179 also inhibited insulin-stimulated NADPH oxidase-mediated  $O_2^{\cdot-}$  production in a dose-dependent way, reaching a significant effect at a concentration of 50  $\mu\text{mol/L}$  (Figure S1). The EC<sub>50</sub> of this metabolite for NADPH oxidase-dependent  $O_2^{\cdot-}$  production was 80  $\mu\text{mol/L}$ . In contrast, EXP3174 did not influence insulin-stimulated NADPH oxidase-dependent  $O_2^{\cdot-}$  production, and losartan exerted an inhibitory effect that only was statistically significant at a very high dose (500  $\mu\text{mol/L}$ ).

To examine whether EXP3179 inhibited  $O_2^{\cdot-}$  production stimulated by another agonist that is known to activate NADPH oxidase through PKC, PBMCs were stimulated with 50 ng/ $\mu\text{L}$  of tumor necrosis factor- $\alpha$ . Interestingly, tumor necrosis factor- $\alpha$ -stimulated  $O_2^{\cdot-}$  production was significantly reduced in the presence of 100  $\mu\text{mol/L}$  of EXP3179 (Figure S2).

Finally, and to test the effect of EXP3179 on NADPH oxidase activity in vascular cells, several experiments were performed in the human endothelial cell line, Eahy926. Interestingly, 100  $\mu\text{mol/L}$  of EXP3179 significantly ( $P < 0.01$ ) reduced PMA-induced NADPH oxidase activity (Figure S3).

To test whether EXP3179 could act as an  $O_2^{\cdot-}$  scavenger, we determined  $O_2^{\cdot-}$  production in a protein homogenate in the absence and presence of EXP3179. Given that  $O_2^{\cdot-}$  levels were not diminished in the presence of EXP3179 (Figure S4), we can discard a relevant role of EXP3179 as an  $O_2^{\cdot-}$  scavenger.



**Figure 1.** Effects of (A) EXP3179, (B) EXP3174, and (C) losartan on PMA-induced phagocytic NADPH oxidase-dependent  $\text{O}_2^-$  production. Phagocytic cells ( $4 \times 10^5$  PBMCs) were stimulated with  $3.2 \mu\text{mol/L}$  of PMA in the absence or presence of different concentrations of losartan and its metabolites. Values are mean  $\pm$  SEM from 25 apparently healthy subjects. \* $P < 0.05$  vs response to PMA.

### EXP3179 Metabolite Blocks p47phox Translocation

An important early step in the activation of the phagocytic NADPH oxidase is the translocation of the cytosolic subunits to membranes. Thus, after PMA stimulation, an increased signal for the p47phox protein expression in the membrane was observed (Figure 3). However, when cells were preincubated with EXP3179, the p47phox translocation induced by PMA was inhibited ( $P < 0.05$ ), thus suggesting that the EXP3179 metabolite may interfere with some of the mechanisms involved in this necessary step for the activation of the NADPH oxidase.

### EXP3179 Metabolite Inhibits PKC

Given that PKC is a relevant activator of NADPH oxidase in phagocytic cells,<sup>3</sup> the effect of EXP3179 on PMA-stimulated PKC activity was analyzed in PBMCs. As shown in Figure 4, PKC activity was enhanced in PMA-stimulated cells. Interestingly, EXP3179 completely inhibited ( $P < 0.05$ ) PKC activity stimulated by PMA in these cells. Thus, we investigated the capacity of EXP3179 to bind to human recombinant PKC $\alpha$  by surface plasmon resonance. As shown in Figure 5A, binding of EXP3179 to PKC $\alpha$  is clear and concentration dependent. We plotted the different EXP3179 concentrations

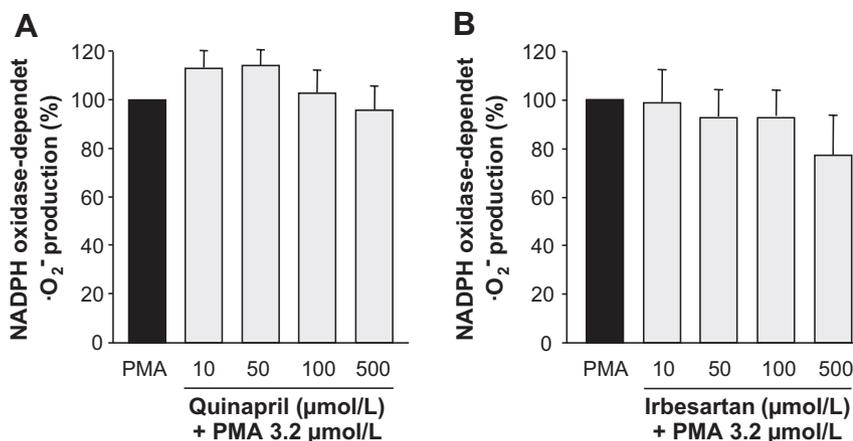
against the corresponding binding responses at equilibrium. To analyze whether the physical interaction between EXP3179 and PKC $\alpha$  would exert a functional effect, we studied the effect of EXP3179 in enzymatic assays and we observed that EXP3179 inhibited ( $P < 0.05$ ) the kinase activity of recombinant PKC $\alpha$ . The inhibitory effect of EXP3179 on PKC activity was in the same range as those observed for bisindolyl maleimide (Figure 5B). Additional experiments demonstrated that EXP3179 also interacted with the PKC $\beta$  isoform and reduced its enzymatic activity (Figure S5).

### EXP3179 Metabolite Blocks MMP-9 Secretion in Phagocytic Cells

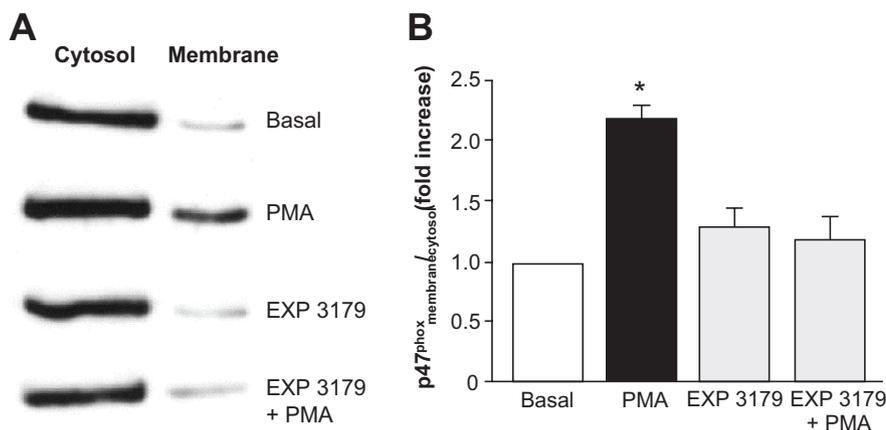
The effect of EXP3179 on MMP-9 secretion was analyzed in human monocytes and PBMCs. EXP3179 inhibited ( $P < 0.05$ ) PMA-induced MMP-9 secretion in the 2 types of cellular preparations (Figure 6).

### NADPH Oxidase-Mediated $\text{O}_2^-$ Production and MMP-9 Levels Are Reduced in Losartan-Treated Hypertensive Patients

The demographic and clinical characteristics of the patients included in this study are summarized in the Table. No differences in blood pressure and other general clinical and



**Figure 2.** Effects of (A) quinapril and (B) irbesartan on PMA-induced phagocytic NADPH oxidase-dependent  $\text{O}_2^-$  production. Phagocytic cells ( $4 \times 10^5$  PBMCs) were stimulated with  $3.2 \mu\text{mol/L}$  of PMA in the absence or presence of different concentrations of both antihypertensive drugs. Values are mean  $\pm$  SEM from 10 apparently healthy subjects. \* $P < 0.05$  vs response to PMA.



**Figure 3.** Effect of EXP3179 on PMA-induced p47phox translocation. Human PBMCs were preincubated for 20 minutes with 100  $\mu\text{mol/L}$  of EXP3179 before 3.2  $\mu\text{mol/L}$  of PMA stimulation. A, A representative immunoblot for the p47phox protein on the cytosol and membrane fractions in the different experimental conditions. B, Densitometric analysis revealed that EXP3179 totally blocked the PMA-induced p47phox translocation. Data are mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs the other conditions.

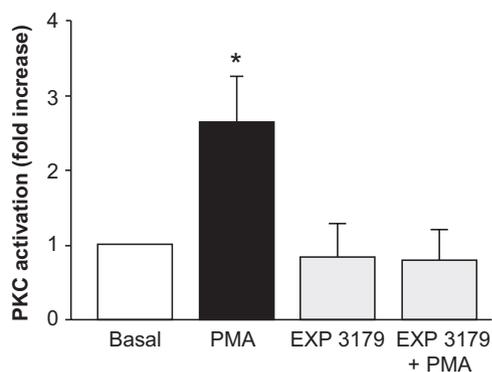
biochemical parameters were found among the 3 subgroups of treated patients.

NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production induced by PMA was lower ( $P < 0.05$ ) in patients treated with losartan than in untreated hypertensives and patients treated with other ARAs or with ACEIs (Figure 7A). PMA-induced NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production measured in patients treated with other ARAs or with ACEIs was similar to that measured in untreated patients (Figure 7A).

Plasma levels of MMP-9 were lower ( $P < 0.05$ ) in patients treated with losartan than in untreated patients and patients treated with other ARAs or with ACEIs (Figure 7B). In addition, plasma levels of MMP-9 measured in patients treated with other ARAs or with ACEIs were similar to those measured in untreated patients (Figure 7B). A positive correlation ( $P < 0.01$ ) was observed between plasma levels of MMP-9 and NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production in the overall population (Figure 7C), which remained significant after controlling for age and sex ( $r = 0.340$ ;  $P < 0.01$ ).

## Discussion

The major findings of this study are that EXP3179 inhibits NADPH oxidase and PKC activities, as well as MMP-9 secretion, in stimulated human phagocytic cells. In addition, NADPH oxidase activity and plasma levels of MMP-9 were lower in hypertensive patients treated with losartan than in

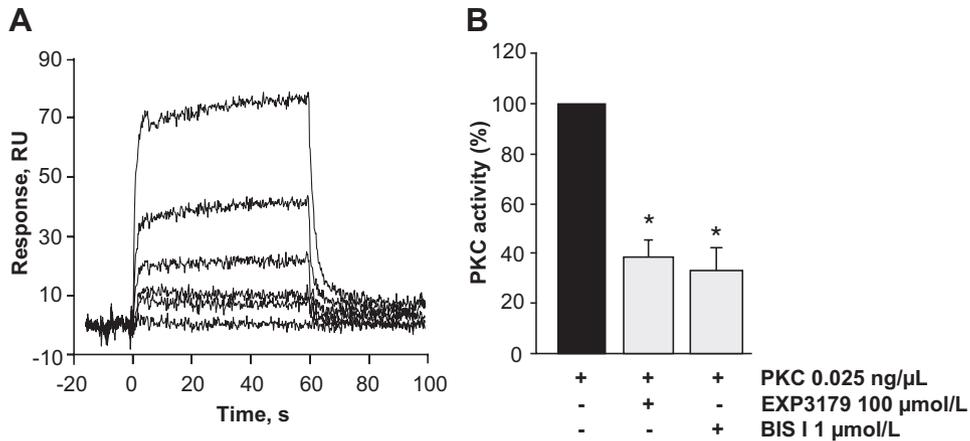


**Figure 4.** Effect of EXP3179 on PMA-stimulated PKC activity. PMA-stimulated PKC activity was totally inhibited with 100  $\mu\text{mol/L}$  of EXP3179 in human PBMCs. Data are mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs the other conditions.

untreated hypertensive patients and hypertensive patients treated with other ARAs and with ACEIs.

It has been proposed that inhibition of phagocytic NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production may represent a novel pharmacological target in the treatment of cardiovascular diseases.<sup>20–22</sup> In this regard, we described for the first time that EXP3179 inhibits the NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production in phagocytic cells. Several arguments suggest that this effect of EXP3179 is independent of the  $\text{AT}_1\text{R}$ . First, the pharmacological profile of losartan metabolites indicates that the EXP3179 is not related to the antagonism of the  $\text{AT}_1\text{R}$ .<sup>13,14,23</sup> Second, neither losartan nor EXP3174 inhibited NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production in phagocytic cells. Lastly, neither irbesartan nor quinapril modified the production of  $\text{O}_2^{\cdot-}$  by NADPH oxidase in these cells.

On cell stimulation, the NADPH oxidase cytosolic subunit p47phox becomes phosphorylated and migrates to the membrane where it binds to the cytochrome, thus activating the oxidase to produce  $\text{O}_2^{\cdot-}$ .<sup>3</sup> Our finding that EXP3179 completely inhibited p47phox translocation from the cytosolic fraction to the membrane fraction further supports the possibility that EXP3179 inhibits PMA-dependent  $\text{O}_2^{\cdot-}$  production by blocking the assembly of NADPH oxidase subunits in phagocytic cells. One of the major mechanisms involved in the phosphorylation and the translocation of NADPH oxidase cytosolic subunits involves the activation of PKC.<sup>3,24</sup> Thus, our finding that EXP3179 inhibits PKC stimulation in phagocytic cells suggests that EXP3179 is inhibiting NADPH oxidase by blocking the PKC signaling pathway. In agreement with this, the surface plasmon resonance data show that EXP3179 interacts with  $\text{PKC}\alpha$ , and the enzymatic assay data indicate that this metabolite inhibits  $\text{PKC}\alpha$ . Collectively, these observations allow us to speculate that PKC may be the target enzyme involved in the ability of EXP3179 to inhibit NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production in phagocytic cells. Although several PKC isoforms have been implicated in signaling responses of phagocytic cells,<sup>25</sup>  $\text{PKC}\alpha$  plays a selective role in PMA-stimulated phagocytic NADPH oxidase-mediated  $\text{O}_2^{\cdot-}$  production.<sup>26</sup> Nevertheless, our results showing that EXP3179 also interacts with  $\text{PKC}\beta$ , which is also activated by PMA in phagocytic cells,<sup>26</sup> allow us to suggest that the inhibitory effect of EXP3179 on the PKC in phagocytic cells is not directed through the specific inhibition



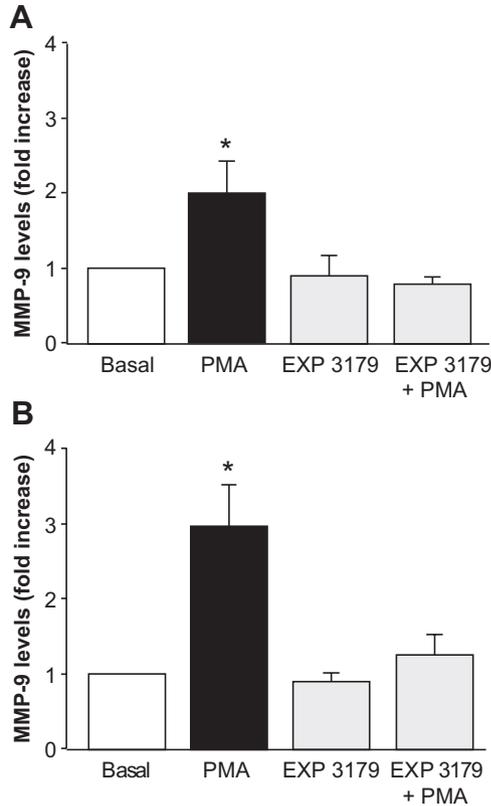
**Figure 5.** EXP3179 interaction with PKC $\alpha$ . A, Several concentrations of EXP3179 (0, 2, 4, 8, 16, and 32  $\mu\text{mol/L}$ ) were injected over a 21 000-RU surface of recombinant PKC $\alpha$ . Sensorgrams show the binding responses of a representative experiment. RU indicates relative units. B, Effect of 100  $\mu\text{mol/L}$  of EXP3179 and 1  $\mu\text{mol/L}$  of bisindolyl maleimide (BIS I) on enzymatic activity of human recombinant PKC $\alpha$ . Data are mean $\pm$ SEM from 5 independent experiments. \* $P<0.05$  vs control conditions.

of only a PKC isoform. Whether the EXP3179 may also interact with other PKC isoforms need to be determined in further experiments.

Given that EXP 3179 presents various potentially vasoprotective effects,<sup>15–18</sup> it is possible to speculate that the pleiotropic actions of EXP3179 occur by a mechanism involving the inhibition of PKC in other vascular cell types. PKC signaling pathways mediate vascular effects, such as the

cyclooxygenase-2 expression,<sup>27</sup> endothelial NO synthase stimulation,<sup>28</sup> regulation of peroxisome proliferator-activated receptor,<sup>29</sup> and platelet activation.<sup>30</sup> Additional studies will be needed to establish the relationship between EXP3179 and PKC in these vascular cell types. Interestingly, our findings show that EXP3179 inhibits the NADPH oxidase activity in endothelial cells. In fact, the activation of the NADPH oxidase in vascular cells has been shown to also be mediated through PKC-dependent pathways.<sup>31</sup>

Our group has demonstrated recently that increased synthesis and secretion of MMP-9 may be consequences of NADPH oxidase-dependent  $\text{O}_2^{\cdot-}$  production in phagocytic cells.<sup>19</sup> In this context, we show that PMA-stimulated MMP-9 secretion by phagocytic cells is inhibited in the presence of EXP3179. The potential relevance of this finding is related to both the fact that MMP-9 has been found to be colocalized

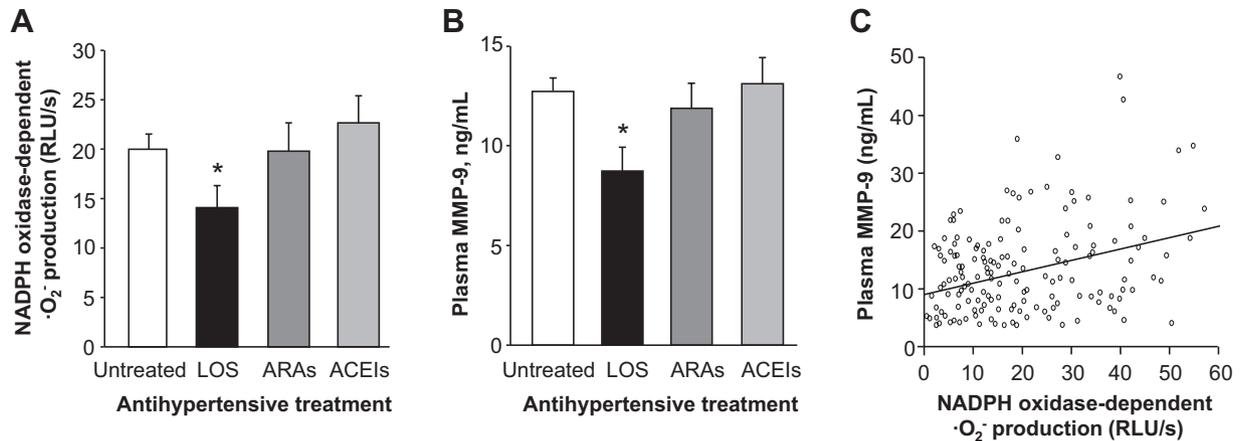


**Figure 6.** Effect of EXP3179 on PMA-induced MMP-9 secretion. PMA-stimulated MMP-9 secretion was completely inhibited by 100  $\mu\text{mol/L}$  of EXP3179 in both (A) human monocytes and (B) PBMCs. Data are mean $\pm$ SEM from 5 independent experiments. \* $P<0.05$  vs the other conditions.

**Table. Demographic and Clinical Characteristics of the Subjects Included in the Study**

Patients Characteristics	Untreated	Losartan	Other ARAs	ACEIs
Age, y	55 $\pm$ 1	62 $\pm$ 2	57 $\pm$ 2	64 $\pm$ 2
Sex, male/female	63/13	18/2	19/7	27/4
Smoking, %	25	35	19	23
Body mass index, kg/m <sup>2</sup>	30.0 $\pm$ 0.5	28.0 $\pm$ 0.6	29.9 $\pm$ 0.8	28.2 $\pm$ 0.7
Systolic blood pressure, mm Hg	145 $\pm$ 1	140 $\pm$ 4	133 $\pm$ 3*	139 $\pm$ 3
Diastolic blood pressure, mm Hg	90 $\pm$ 1	83 $\pm$ 2*	83 $\pm$ 1*	82 $\pm$ 1*
Mean blood pressure, mm Hg	127 $\pm$ 1	120 $\pm$ 2*	115 $\pm$ 2*	119 $\pm$ 2*
Total cholesterol, mmol/L	6.0 $\pm$ 0.1	5.2 $\pm$ 0.2*	5.4 $\pm$ 0.2*	5.3 $\pm$ 0.2*
LDL cholesterol, mmol/L	4.1 $\pm$ 0.1	3.3 $\pm$ 0.1*	3.5 $\pm$ 0.1*	3.5 $\pm$ 0.1*
HDL cholesterol, mmol/L	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1
Glucose, mg/dL	104 $\pm$ 2	104 $\pm$ 4	109 $\pm$ 5	115 $\pm$ 6
Triglycerides, mmol/L	1.4 $\pm$ 0.1	1.2 $\pm$ 0.1	1.5 $\pm$ 0.2	1.4 $\pm$ 0.1
MMP-9, ng/mL	12.7 $\pm$ 0.8	8.8 $\pm$ 1.0*	11.9 $\pm$ 1.2	13.1 $\pm$ 1.2

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein. \* $P<0.05$  vs nontreated antihypertensive patients.



**Figure 7.** Effects of different antihypertensive treatments on (A) phagocytic NADPH oxidase–dependent  $\text{O}_2^-$  production and (B) plasma levels of MMP-9 in hypertensive patients. Hypertensive patients were classified into 4 groups: untreated patients ( $n=76$ ), patients treated with losartan (LOS;  $n=20$ ), patients treated with other ARAs ( $n=26$ ), and patients treated with ACEIs ( $n=31$ ). RLU/s indicates relative light units per second. Data are mean  $\pm$  SEM. \* $P < 0.05$  vs the other conditions. C, Positive correlation between NADPH oxidase–dependent  $\text{O}_2^-$  production and plasma levels of MMP-9 ( $y = 9.24 + 0.19x$ ;  $r = 0.340$ ;  $P < 0.01$ ) in the whole hypertensive population.

with NADPH oxidase components in macrophage-rich areas in endarterectomies from atherosclerotic patients<sup>19</sup> and the findings that support a role for MMP-9 in the development of the plaque.<sup>32,33</sup>

The clinical meaning of the in vitro observations here reported is underlined by our observations in hypertensive patients. We show, for the first time, that patients treated with losartan exhibit lower values of NADPH oxidase–dependent  $\text{O}_2^-$  production than untreated hypertensives. Because no changes in this parameter were observed either in patients treated with other ARAs or in patients treated with ACEIs, we can speculate that the observed effect in losartan-treated patients is independent of angiotensin II and the  $\text{AT}_1\text{R}$ , thus reinforcing the potential contribution of EXP3179 to the above findings.

Finally, we found that hypertensive patients treated with losartan exhibited lower plasma levels of MMP-9 than untreated hypertensives and hypertensive patients treated with other ARAs or ACEIs. In addition, we found a significant positive bivariate correlation between phagocytic NADPH oxidase–dependent  $\text{O}_2^-$  production and plasma levels of MMP-9 in the whole population of hypertensive patients. Nevertheless, we cannot discard the contribution of other vascular cells, including endothelial cells and smooth muscle cells, on the generation of MMP-9.<sup>34,35</sup> Collectively, these findings support the possibility that reduced MMP-9 might be an additional beneficial effect of losartan in hypertension, likely attributed to the ability of its EXP3179 metabolite to inhibit phagocytic NADPH oxidase–dependent  $\text{O}_2^-$  production. This specific property of losartan may be involved in its capacity to inhibit experimental atherosclerosis,<sup>36</sup> as well as to prevent ischemic atherosclerotic events in hypertensive patients.<sup>37</sup>

### Perspectives

Albeit preliminary, these data suggest that EXP3179 acts as an inhibitor of NADPH oxidase, namely in phagocytic cells, by a potential mechanism that targets the PKC signaling pathway. Furthermore, the observed effects of EXP3179 on

MMP-9 secretion from phagocytic cells might be downstream effectors of PKC inhibition. Thus, it is proposed that EXP3179 can be a specific mediator of the ability of losartan to reduce cell-mediated oxidative stress in hypertension.

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### Disclosures

None.

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**ONLINE SUPPLEMENT**

**Losartan metabolite EXP3179 blocks phagocytic NADPH oxidase-mediated superoxide production by inhibiting PKC. Potential clinical implications in hypertension.**

Short title: EXP3179 and NADPH oxidase in hypertension.

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## EXPANDED METHODS

### NADPH oxidase activity in endothelial cells

In order to analyze the effect of the EXP3179 on NADPH oxidase activity in non-phagocytic cells, endothelial cell line (Eahy926) was employed. Cells were maintained in DMEM supplemented with fetal calf serum (FCS) 10%, glutamine 4 mol/L, sodium pyruvate 1 mmol/L, penicillin 100 U/mL and streptomycin 100 µg/mL to reach 80-90% confluence at 37 °C in a humidified environment with 5% CO<sub>2</sub>. Before four hours of stimulation with PMA 3.2x10<sup>-6</sup> mol/L, cells were preincubated with EXP3179 100 µmol/L during 30 min. Cells were then scrapped and homogenized on ice. Protein concentration was measured by using the Lowry method.

Chemiluminescence assays with lucigenin 5 µmol/L and NADPH 0.1 mmol/L were used to measure NADPH oxidase activity in 15 µg of protein homogenates. The reaction was started by addition of lucigenin to protein homogenates in a final volume of 300 µL. Luminescence was measured 5 minutes in a luminometer. A buffer blank was subtracted from each reading. The value of the area under the curve was used to quantify chemiluminescence. Data are expressed as relative light units produced per second.

### p47phox translocation experiments

In order to analyze the effect of the EXP3179 on a direct index of NADPH oxidase activation, we analyzed the effect of this metabolite on the translocation of p47phox from cytosol to membranes. PBMCs (5x10<sup>6</sup> cells) were stimulated with PMA 3.2 µmol/L in the presence (20 minutes of preincubation) of EXP3179 100 µmol/L. After 15 minutes cells were centrifuged at 200g for 5 minutes and resuspended in 1 mL of Tris buffer saline (Tris 10 mmol/L pH 8, NaCl 150 mmol/L) containing a protease inhibitor cocktail Complete (Roche). Then, cells were centrifuged at 2000g for 10 minutes. The supernatant was collected and centrifuged at 100000g for 60 minutes at 4°C. The supernatant, which corresponded to the cytoplasmatic fraction, was mixed with loading buffer (Tris 125 mmol/L, SDS 4%, glycerol 20%, β-Mercaptoethanol 10% and Bromophenol Blue 0.0125%). The pellet, which corresponded to the membrane fraction, was resuspended with loading buffer in the same volume as cytoplasmatic fraction.

p47phox subunit expression was quantified by Western blot as previously published.<sup>1</sup> Expression of the housekeeping protein β-actin was also evaluated. Antibodies were purchased from Santa Cruz Biotechnology.

### Determination of MMP-9 levels

A sandwich ELISA (Amersham Biosciences) was used to determine MMP-9 levels in plasma samples from the patients and in cultured cells supernatant as previously published.<sup>2</sup> Briefly, MMP-9 secretion was analyzed both in human PBMCs (1.25x10<sup>6</sup> cells) or in monocytes (2.5x10<sup>5</sup> cells) previously isolated from PBMCs by using the MACS Column Technology and CD14 MicroBeads (Miltenyi Biotech). In both cases, cells were plated in a 96-well plate for 16 hours. Cells were preincubated for 20 minutes with EXP3179 100 µmol/L before stimulation with PMA 64 nmol/L. MMP-9 was determined in the supernatants 24 hours later.

### Determination of PKC activity

PKC activity was determined by a non-radioactive ELISA (Assay Designs) in PMA-stimulated cellular homogenates of PBMCs. PBMCs ( $5 \times 10^6$  cells) were preincubated for 20 minutes with EXP3179 100  $\mu\text{mol/L}$  before stimulation with PMA 64  $\text{nmol/L}$ . After 10 minutes of stimulation, cells were centrifuged at 300g for 10 minutes at  $4^\circ\text{C}$ , and washed with cold PBS pH 7.4. After adding 300  $\mu\text{L}$  of lysis buffer (MOPS 20  $\text{mmol/L}$ ,  $\beta$ -glycerolphosphate 50  $\text{mmol/L}$ , sodium fluoride 50  $\text{mmol/L}$ , sodium vanadate 1  $\text{mmol/L}$ , EGTA 5  $\text{mmol/L}$ , EDTA 2  $\text{mmol/L}$ , NP40 1%, dithiothreitol 1  $\text{mmol/L}$ , benzamidine 1  $\text{mmol/L}$ , phenylmethanesulphonylfluoride 1  $\text{mmol/L}$ , leupeptin 10  $\mu\text{g/mL}$  and aprotinin 10  $\mu\text{g/mL}$ ) containing a protease inhibitor cocktail Complete (Roche), the mixture were incubated for 10 minutes on ice. After centrifugation at 15000g for 15 minutes at  $4^\circ\text{C}$ , the supernatant, that constituted the cytosolic fraction, was transferred to a pre-chilled tube to be stored at  $-70^\circ\text{C}$ .

By using the same ELISA, we studied the inhibitory effect of EXP3179 on the enzymatic activity of the human recombinant PKC $\alpha$  and PKC $\beta$  (SIGMA). In this study, the specific inhibitor of PKC, bisindolyl maleimide (BIS) I 1  $\mu\text{mol/L}$ , was used as a positive control.

### Biomolecular interactions analysis.

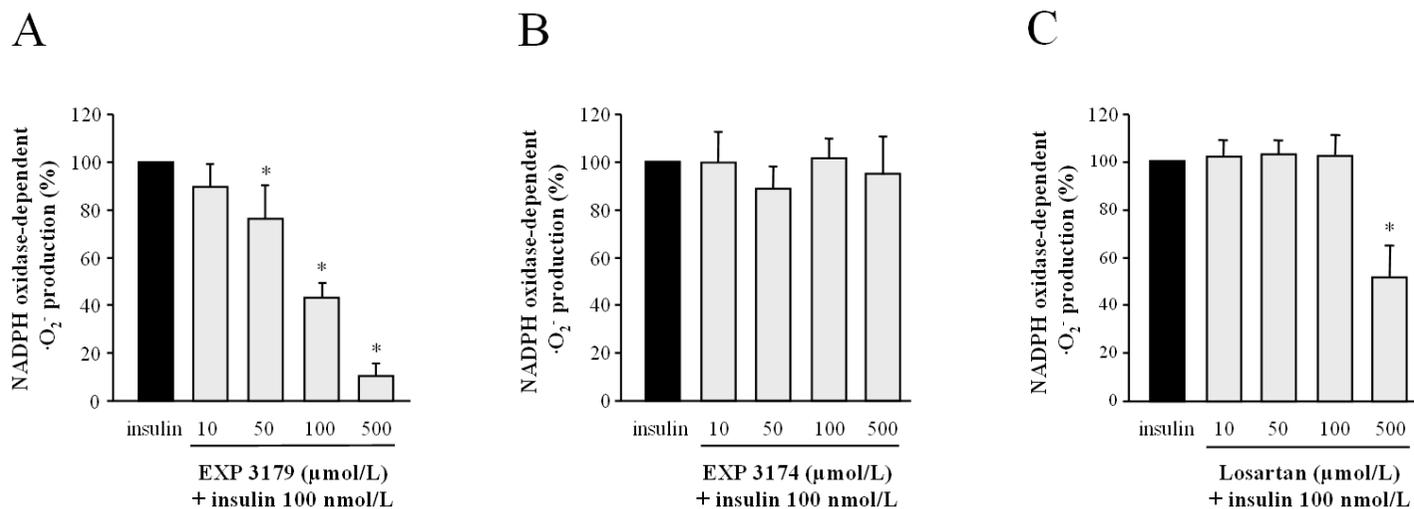
Interaction experiments were performed using surface plasmon resonance (SPR) technology in a BIAcore X Biosensor. A PKC $\alpha$  surface (21,000 RU) was prepared with the amine coupling Kit. Anti- $\beta$ 2 microglobulin antibody was immobilized on flow channel 1 in order to create a reference surface. EXP3179 at concentrations of 0, 2, 4, 8, 16 and 32  $\mu\text{mol/L}$  was injected in duplicate in HEPES 10  $\text{mmol/L}$ , NaCl 150  $\text{mmol/L}$ , Tween-20 0.005% (v/v) (pH 7.4) at a flow of 30  $\mu\text{L/min}$  and allowing an association time of 60 s. Mass transport limitation effects were excluded. BIS I 50  $\mu\text{mol/L}$  was also injected onto the PKC surface and a clear and specific binding signal was detected (data not shown). Thus, this system is suitable for checking the interaction between PCK and its ligands. Kinetic and affinity analysis were performed using BIAEVALUATION software 3.2RC1. Finally, we analyzed also the interaction of human recombinant PKC $\beta$  (21,000 RU) with EXP3179 32  $\mu\text{mol/L}$ .

### Statistical analysis

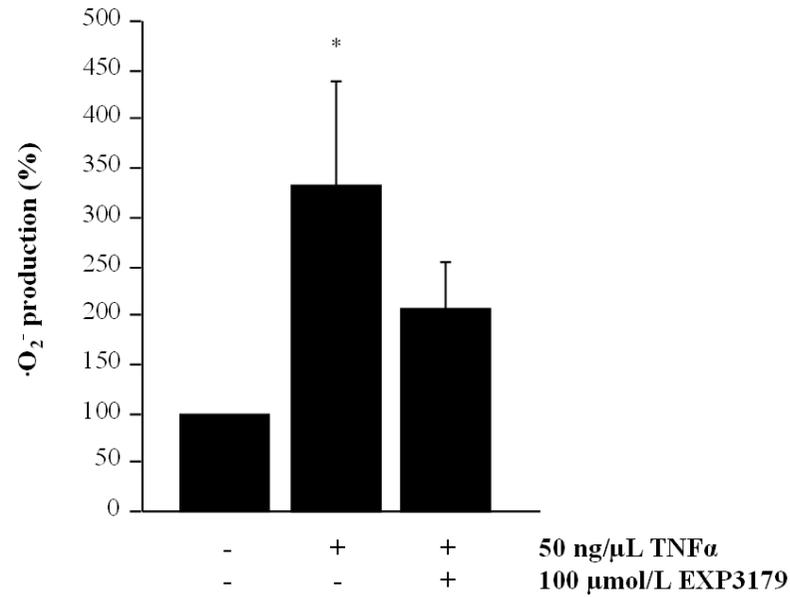
Data are expressed as mean $\pm$ SEM. Differences among groups were assessed by one-way ANOVA once normality had been demonstrated and a Scheffé *post hoc* test was used to examine differences between groups when significance was achieved; otherwise a Kruskal-Wallis followed by a Mann-Whitney U test was used. Pearson correlation test was used to assess correlation between plasma MMP-9 levels and NADPH oxidase-dependent  $\cdot\text{O}_2^-$  production. The calculations were performed using the SPSS/Windows version 13.0 statistical package. Statistical significance was established as  $P < 0.05$ .

### REFERENCES

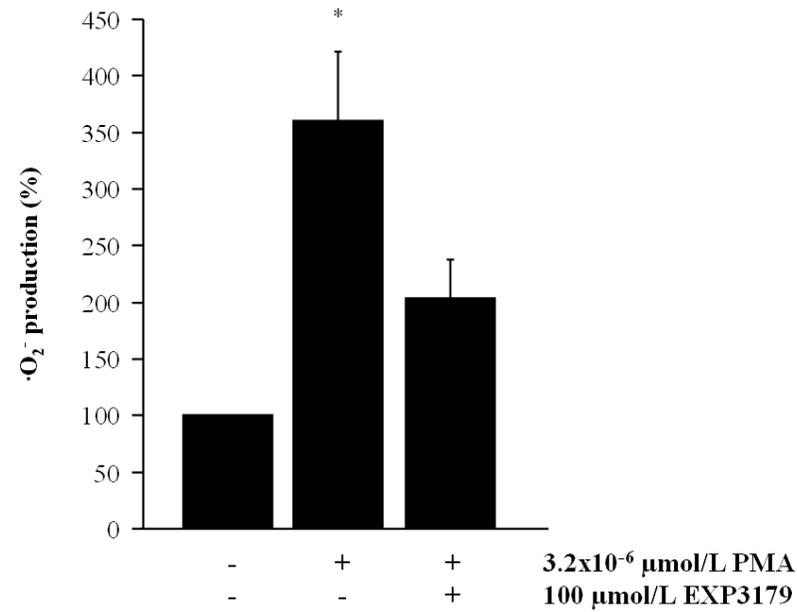
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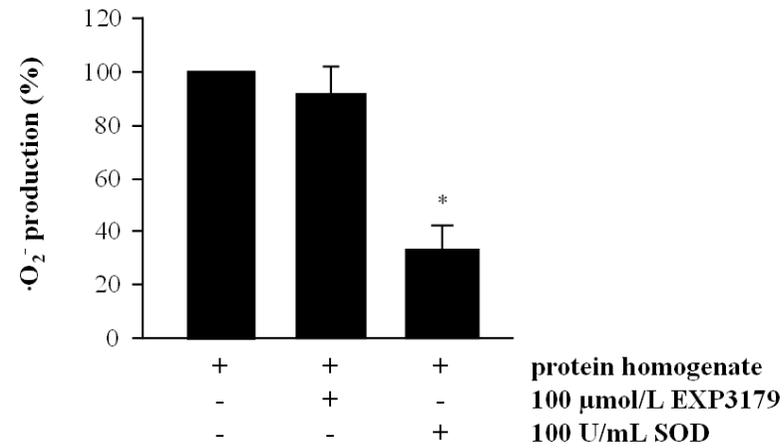
**Figure S1.** Effects of (A) EXP3179, (B) EXP3174 and (C) losartan on insulin-induced phagocytic NADPH oxidase-dependent  $\cdot\text{O}_2^-$  production. Phagocytic cells ( $4 \times 10^5$  PBMCs) were stimulated by 100 nmol/L insulin in the absence or presence of different concentrations of losartan and its metabolites. Values are mean  $\pm$  SEM from 10 healthy subjects. \* $P < 0.05$  vs response to insulin.



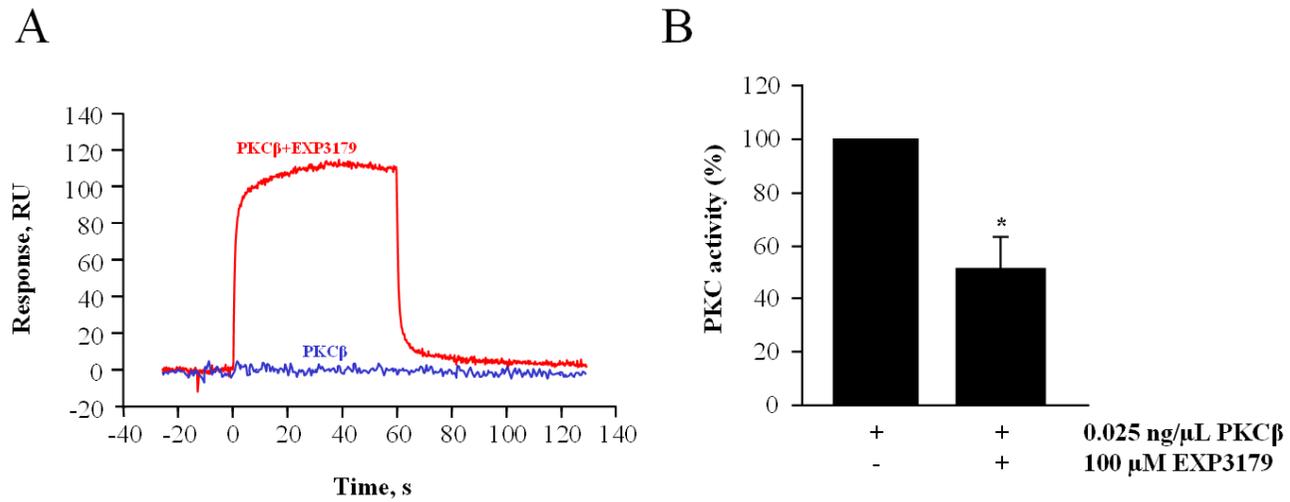
**Figure S2.** Effect of EXP3179 on TNF $\alpha$ -induced phagocytic NADPH oxidase-dependent  $\cdot\text{O}_2^-$  production. Phagocytic cells ( $4 \times 10^5$  PBMCs) were stimulated with TNF $\alpha$  50 ng/ $\mu\text{L}$  in the absence or presence of EXP3179 100  $\mu\text{mol/L}$ . Values are mean $\pm$ SEM from 7 healthy subjects. \* $P < 0.05$  vs other conditions.



**Figure S3.** Effect of EXP3179 on NADPH oxidase activity determined in Eahy926 endothelial cell line. Eahy926 cells were stimulated with PMA in the absence or presence of EXP3179 100 μmol/L. Superoxide production was measured with NADPH 100 μmol/L in the presence of lucigenin 5 μmol/L, in 15 μg of protein homogenates. Values are mean±SEM from 5 independent experiments. \**P*<0.05 vs other conditions.



**Figure S4.** Effects of EXP3179 100 μmol/L and SOD 100 U/mL on  $\cdot\text{O}_2^-$  production from a protein homogenate of rat kidney. Values are mean±SEM from 5 independent experiments. \* $P$ <0.05 vs other conditions.



**Figure S5.** Effect of EXP3179 on human recombinant PKC $\beta$ . (A) Red sensogram shows the binding response between the PKC $\beta$  and the EXP3179. RU, Relative Units. (B) Effect of 100  $\mu$ mol/L EXP3179 on enzymatic activity of PKC $\beta$ . Data are mean $\pm$ SEM from 3 independent experiments. \* $P$ <0.05.