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PKCε-mediates resistin-induced NADPH oxidase activation and inflammation leading to smooth muscle cell dysfunction and intimal hyperplasia

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Abstract

Backgrund and aims—Resistin has been implicated in cardiovascular disease and poor interventional cardiovascular outcomes. Previous studies by our group demonstrated resistin promoted vascular smooth muscle cell (VSMC) migration through protein kinase C epsilon (PKCε) pathways, while few others showed that resistin induced reactive oxygen species (ROS) generation in various cell types. In this study, we aim to systemically examine the functional role of resistin at the cellular and tissue levels as well as the potential mechanistic relationship between resistin-induced PKCε activation and ROS production.

Methods—Plasma collected from patients undergoing carotid interventions was analyzed for resistin level and ROS. VSMCs were treated with resistin in the presence or absence of PKCε and NADPH oxidase (Nox)-specific inhibitors. Intracellular ROS production was analyzed using confocal microscopy and Nox activity with chemiluminescence. *In vivo* studies were performed in apolipoprotein E knock out ($ApoE^{-/-}$) mice to determine therapeutic effects of PKCε-specific inhibitor, using the guide-wire injury model.

Results—We observed significant correlation between plasma resistin and circulating levels of oxidative stress in patients with severe atherosclerotic disease. We also demonstrated that resistin induced ROS production via PKCε-mediated Nox activation. Resistin-induced ROS production was time-dependent, and Nox4 was the primary isoform involved. Inhibition of Nox completely abolished resistin-exaggerated VSMC proliferation, migration and dedifferentiation, as well as pro-inflammatory cytokine release. Upstream modulation of PKCε significantly reduced resistin-mediated cytosolic ROS, Nox activity and VSMC dysfunction. Moreover, PKCε-specific inhibitor mitigated resistin-induced Nox activation and intimal hyperplasia in $ApoE^{-/-}$ mice.

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Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Conclusions—Resistin-associated VSMC dysfunction and intimal hyperplasia are related to PKCε-dependent Nox activation and ROS generation. Targeting the PKCε-Nox pathway may represent a novel strategy in managing resistin-associated atherosclerotic complications.

Keywords

resistin; protein kinase C epsilon; NADPH-oxidase; vascular smooth muscle cell; inflammation; intimal hyperplasia

Introduction

Resistin, an adipokine expressed primarily in cells of monocyte/macrophage lineage in humans, is elevated in obese and diabetic individuals[1,2] and has been associated with cardiovascular disease (CVD) and poor clinical outcomes.[3] Serum resistin levels increase with exacerbated coronary artery disease (CAD) in patients with carotid artery stenosis.[4] Additionally increasing clinical evidence shows that resistin is an independent predictor of major adverse cardiovascular events including restenosis, myocardial infarction, and death in patients undergoing coronary interventions.[5] Elevated plasma resistin has been associated as a risk factor for CVD in a European population study and shown to be high in patients with carotid artery disease,[6] but some studies did not find an association.[7,8] Abundant resistin has also been detected in atherosclerotic regions of the human carotid artery and aorta.[9] There is thus a large body of clinical data suggesting a potential role of resistin in atherosclerosis. However, direct evidence on the effect of resistin on clinical outcomes is still largely unexplored.

Controversial findings with respect to resistin's mode of action in different species and cells types exist.[10,11] However increasing evidence suggests that its inflammatory properties may be responsible for its effects in the human vasculature and atherosclerosis.[12] Resistin has several features in common with pro-inflammatory cytokines.[13,14] It promotes inflammation through induction of other cytokines, and the expression of resistin itself is up-regulated in peripheral blood mononuclear cells in response to stimulation by pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β, and LPS.[15–16] In humans, elevated levels of resistin are frequently found in association with autoimmune diseases and inflammation.[17,18] Resistin at a pathological concentration promotes vascular cell dysfunction, which includes endothelial cell activation,[18] monocyte-endothelial cell adhesion,[19] and vascular smooth muscle cell (VSMC) proliferation and migration.[20–22] However, the underlying mechanisms of resistin-induced cytokine secretions and whether the cellular effect can be translated *in vivo*, are largely unknown.

We previously have demonstrated that protein kinase C epsilon (PKCε) is a novel upstream modulator for resistin-induced VSMC migration.[20] Others have shown that resistin induces ROS production in various cell types.[23–24] We hypothesized that PKCε is a key mediator for resistin-stimulated ROS production and subsequent inflammation and cellular dysfunction in VSMCs. In this study, we determined the role of resistin in atherosclerois through mechanistic evaluations, by: 1) examining relationships between ROS and resistin in human

plasma; 2) performing *in vitro* studies in a human VSMC model; and 3) verifying the applicability of our observations in an *in vivo* gene knockout murine model.

Materials and methods

Human plasma analysis

Human plasma were collected from 99 elderly patients (mean age: 69.3 years) who underwent carotid interventions following an established protocol (IRB 23476). Samples were stored at −80 °C and analyzed with a Luminex magnetic beads-based assay for circulating resistin levels. Plasma carbonyl levels were measured as described later in this section.

Cell culture and in vitro treatments

Human coronary artery smooth muscle cells (HCASMC) or VSMCs from Genlantis[25] were used at passage 5 to 8 for experiments. We chose a pathological resistin level of 40 ng/mL for our studies, based on published reports of resistin in human subjects.[25–28] Cells were treated for various time points with or without resistin at 40 ng/mL in the presence or absence of 1 μM PKCε-specific peptide inhibitor, εV1-2, or Nox inhibitors VAS-2870 (10 μM) and DPI (5 μM). Inhibitors of other oxidases: rotenone (mitochondrial electron transport chain), and allopurinol (xanthine oxidase) were both used at 1 μM. Cells were pre-treated with inhibitors for 30 min before addition of resistin.

Cytosolic ROS measurement

VSMCs grown on coverslips were either treated with or without resistin and in the presence or absence of inhibitors for the different time points. Cells were incubated with 2 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) in HBSS at 37 °C for 30 min (for cytosolic ROS)[29] and imaged using confocal microscopy (ZEISS Confocal LSM 710).

Nox activity

Nox activity was measured by the lucigenin-enhanced chemiluminescence method as described.[30] Briefly, cultured VSMCs were homogenized in lysis buffer followed by sonication and centrifugation at 8100*g* at 4°C for 10 min. Nox specific activity was measu red in the presence of VAS-2870 or DPI. Apocynin was not tested because of its reported inactivity in vascular cells.[31] Nox dependent superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/mg of protein. Results are expressed as fold change in Nox activity compared to control.

Real-time polymerase chain reaction (RT-PCR)

Total RNA from VSMCs was isolated using TRIzol according to standard protocols. SYBR green PCR master mix was used for real time PCR. Human primers are listed in Table 1.[32] RT-PCR was performed in a Mastercycler RT-PCR detection system (Eppendorf, Westbury, NY). The relative level of target (Nox isoforms) gene in each group was normalized against internal housekeeping gene *18S* rRNA using the calculation formula of $2^{\Delta Ct[18SrRNA\text{-}Nox]}$.

The target gene levels in drug treated groups were further normalized against the control group.

PKC activity

Total cell lysates were collected and the quantification of PKCε specific activity was carried out using the PKC activity kit (Enzo Life sciences), according to the manufacturer's instructions. Cell samples were assayed in the presence and absence of εV1-2 (1 μM) and the difference in values is used to calculate PKCε-specific activity. The assay was quantified with a spectrophotometric microplate reader (iMark, Bio-Rad) at a dual wavelength of 450/595 nm. Data is presented as amount of active PKC/mg protein.

Cytokine measurement

0.2×10^6 VSMCs were plated in 24 well plates and serum starved overnight. Cells were then treated with resistin (40 ng/ml) for different time periods in the absence or presence of PKCε and Nox inhibitors. Supernatants were collected and analyzed for TNF-α, IL-1β and IL-6 using standard ELISA manufacturer's protocols (Thermo Scientific).

Cell growth for extended time points using growth curves

To assay cell proliferation kinetics, cells were seeded at a density of $3\times10^3/cm^2$ and grown in the same medium and conditions, as described above. Three wells of plated cells per each condition were counted after each time point (0–7 days) on a TC10 automated cell counter (BioRad). Cell number was graphed vs time to obtain the growth curves and exponential growth curve fitting was performed using the Prism 6.0 software (GraphPad Inc).

Cell migration using scratch wound assay

VSMCs were grown to confluence in a 6-well plate. A transverse scratch wound on each monolayer of VSMC was made using a sterilized 200 μl-tip. The scratch wounded monolayers were then stimulated with or without resistin (40 ng/ml) in the presence or absence of εV1-2 (1 μM) and Nox inhibitor VAS-2870 (10 μM) or DPI (5 μM) for an additional 24 h, at which point the transverse scratch wounds were re-examined for cell migration. Pictures were captured with a phase-contrast microscope and migration was quantified using ImageJ software. Cell migration was calculated as the percent of wound closure relative to controls.

Cell dedifferentiation and Western blot

Cell lysate samples (15 μg of protein) separated on a 4–20% polyacrylamide gel for electrophoresis were transferred onto nitrocellulose membranes using Bio-Rad Mini-Trans-Blot system. The blocked membrane was incubated with primary antibodies overnight at 4°C and with secondary antibody for 1 hour at room temperature. The immunoreactive bands were detected using a BIO-RAD chemiluminescence system, and the bands were captured and intensity quantified by with BIO-RAD ChemiDoc XRS+ camera and Image Lab software respectively.

Guide wire injury and ultrasound evaluation

6–8 week old male *ApoE*$^{-/-}$ mice on C57BL/6 background (Jackson Labs) were subjected to guide wire injury as described earlier.[33] The animals were randomly divided into four treatment groups (n=6–8 per group): vehicle control (normal saline), resistin (1 μg/day), εV1-2 (3 mg/kg/day), or combined resistin and εV1-2 (Resistin+εV1-2), and drugs were delivered using an mini-osmotic pump (ALZET, Model 2004, CA) implanted subcutaneously. All mice underwent carotid evaluations using a VisualSonics Vevo 770 high-resolution ultrasound micro-image system and once every 2 weeks afterwards. Diameters of bilateral CCA were measured at distal and proximal CCAs in both longitudinal and transverse views. After four weeks post-injury, the mice were sacrificed and bilateral CCAs were harvested for TBARS and protein carbonyl assay. The animal IACUC protocol (ACORP1480) was approved.

Protein carbonyl assay

Cell extracts were mixed with 1 volume of 20% trichloroacetic acid to precipitate proteins. Carbonyl content was determined by following the standard spectrophotometric assay procedure. The protocol involves measurement of absorbance at 276 nm of the DNPH-derivatized samples in 6 M guanidine, using 50,000 for molar absorptivity and quantifying for protein.[34]

Plasma isoprostane analysis

Plasma from human subjects was purified on SPE cartridges before analysis for isoprostane using the Cayman kit according to the manufacturer's instructions. Isoprostane levels were calculated and expressed in pg/mL.

Statistical analysis

All experiments were performed at least four times (n=4 to 6) in duplicate. The results are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 software. We performed parametric or non-parametric analyses on the data sets and the appropriate statistical test used. Time-dependent data from ROS, PKC activity and cell proliferation were compared by one-factor analysis of variance (ANOVA) followed by Dunnett's test. Effect of inhibitor data from Nox and PKCε inhibitor experiments on ROS and proliferation were analyzed using two-factor ANOVA followed by Tukey's test. Results from migration, Nox activity and real time PCR did not follow a normal distribution and was analyzed using the non-parametric Mann Whitney test. Similarly data from *in vivo* experiments were analyzed using the non-parametric test. Statistical significance was considered if the p-value was < 0.05.

Results

Correlation of systemic resistin levels to ROS

Patients were divided into tertiles based on plasma resistin concentration (n=33 in each group): Low: 2.3 ng/mL (1.0–3.4 ng/mL); Medium: 4.2 ng/mL (3.4–4.9ng/mL); and High: 7.3 ng/mL (4.9–13.3 ng/mL). Circulating plasma carbonyl levels and isoprostane levels

correlate with resistin levels (Fig. 1A), showing that patients with higher circulating resistin levels have higher oxidative stress as shown by significantly higher levels of plasma protein carbonyl (top panel) as well as isoprostane (low panel). The highest circulating level of resistin in our patient cohort is 13ng/mL. However, other studies suggest much greater levels, up to 100ng/mL in laboratory investigations.[21,35] We have also previously shown that 40ng/mL of resistin significantly stimulates VSMC migration. To be consistent with our previous study and others,[20,24] and after evaluating a dose-dependent response curve, we chose 40 ng/ml of resistin for the rest of this study.

Resistin dose, time-dependently induces ROS production

Dose-dependent increases in ROS production was observed in VSMCs within 2 hours of resistin treatment (Fig. 1B).

Significant increase in ROS was evident when VSMCs were treated with as low as 10 ng/mL of resistin. Cytosolic ROS production in VSMCs was seen as early as 30 minutes of resistin (40 ng/mL) treatment and increased in a time-dependent manner up to 24 hours as evidenced from Fig. 1C. Long duration of treatment, up to 7 days, still showed increased ROS levels as compared to untreated controls (data not shown), suggesting that both acute and chronic resistin treatment induces oxidative stress in VSMCs. Specific inhibitors of oxidases *viz.*, rotenone (200 nM), allopurinol (250 μM), DPI (5 μM), and VAS-2870 (10 μM) were used to identify potential oxidases involved. VAS-2870 completely inhibited resistin-induced ROS production (DPI did the same; data not shown), as measured by fluorescence intensity using a plate reader (Supplementary Fig. 1A and B) and confocal microscopy (Fig. 1D). On the other hand, neither rotenone nor allopurinol had significant effect, suggesting that Nox is the major cytosolic source of ROS, and that SOD or xanthine oxidase were not involved (data not shown). Inhibitor concentrations used were based on the Ki values. For all our experiments both Nox inhibitors (VAS-2870 and DPI) were tested and found to have similar effects although only results from VAS-2870 are shown.

Resistin activates NOX via PKCε

We know from earlier investigations that PKCε mediates VSMC migration triggered by resistin. PKCε activation in VSMCs was evaluated using a PKC activity kit, as well as immunofluorescence detection of phosphorylated PKCε (phosphoS729-PKCε). PKCε specific activity was determined using the PKCε specific inhibitor, εV1-2.[20,36] Fig. 1E shows the time-course of PKCε activation in VSMCs following resistin treatment. Significant increase in activity is observed as early as 5 min and up to 4 hours using a PKC activity kit. Increased phosphorylation of PKCε was observed as early as 10 min, which was inhibited when cells were pre-treated with εV1-2 prior to resistin treatment (Supplementary Fig. 2A and B).

We investigated the role of PKCε in resistin-induced ROS generation and observed that the PKCε inhibitor, εV1-2, completely blocks resistin-induced ROS production. Having identified Nox as the primary oxidase involved in VSMC-resistin interaction, change in chemiluminescence in the presence and absence of VAS-2870 or DPI was used to calculate Nox specific activity in cell homogenates. Resistin (40 ng/mL) significantly induced

activation of Nox compared to the control (17.5 nmol/mg protein/min vs.8.2 nmol/mg protein/min P<0.001) (Fig. 1F). Nox activity was markedly suppressed by εV1-2. Differential regulation of Nox homologues *viz. Nox1*, *Nox2*, *Nox3*, *Nox4* and subunits *p22phox* and *p47phox* in VSMCs were studied by real time PCR. Resistin upregulated the expression of *Nox4* and *p22phox* in VSMCs (Fig. 1G), while the levels of *Nox1*, *Nox2* and the other subunits were unchanged. εV1-2 reversed resistin-associated *Nox4* and *p22phox* up-regulation, suggesting that PKCε is an upstream mediator for resistin-induced Nox activation and ROS production. Involvement of Nox4 was tested using siRNA approach which almost completely quenched resistin induced increase in ROS (Fig. 1H). Efficacy of siRNA inhibition of Nox4 was tested by real time PCR as shown in Supplementary Fig. 3A. Nox 4 siRNA completely inhibited resistin induced proliferation as shown in Supplementary Fig. 3B.

PKCε and Nox modulation mitigates resistin-induced VSMC dysfunction

Crucial functions of VSMC in atherosclerotic plaque formation were evaluated, including migration, proliferation, and dedifferentiation. Resistin treatment increased VSMC migration, and it was suppressed by VAS-2870 and εV1-2 as shown in Fig. 2A (images shown in Supplementary Fig. 4A). The inhibitors alone did not have visible effect on VSMC migration. Cell proliferation was measured using the MTT assay and the growth curve method. Cell viability after 24 hours of resistin treatment was significantly higher (~1.5 fold) than untreated cells (p <0.0001) based on the MTT assay (Supplementary Fig. 4B) and inhibited by ROS scavenger (Supplementary Fig. 5). Growth curve of resistin treated cells also showed increased VSMC growth in time-course ranging from 0 to 7 days (Fig. 2B). Both VAS-2870 and εV1-2 decreased the growth of VSMCs significantly. Most healthy VSMCs *in vivo* exhibit the contractile phenotype while the de-differentiated, synthetic-phenotype is often displayed during atherosclerotic plaque development. De-differentiated VSMCs lose their primary contractile markers including smooth muscle α-actin (α-SMA), smooth muscle myosin heavy chain (SM-MHC), smoothelin, SM22α and calponin-1.[37] We observed that resistin promoted VSMC dedifferentiation after 7 days of treatment (Fig. 2C) as evident by reduction in the contractile phenotype markers following resistin treatment compared to the control. Representative blots for the different proteins and gene expression profile are shown in (Supplementary Fig. 4C and D). Resistin-associated VSMC dedifferentiation was reversed by VAS-2870 and εV1-2. Collectively, the data suggests PKCε and Nox mediate resistin-associated smooth muscle cell dysfunction.

PKCε mediates resistin-induced cytokine release by VSMCs

We see a time dependent increase in TNFα and IL-1β in VSMCs with a peak level reached at 4–8 hours after resistin treatment. Inhibition of PKCε and Nox during the 8 hours of treatment totally abolishes resistin-induced cytokine release as shown in Fig. 3A and C. The trend of IL-6 was however different and was not affected by either inhibitors (Fig. 3B), suggesting that resistin induces acute-phase inflammatory cytokines, but not chronic inflammation in VSMCs.

Resistin exacerbates intimal hyperplasia in denuded mouse carotid arteries

ApoE$^{-/-}$ mice fed on western diet received carotid denudation as previously described.[33] We observed progressive luminal reduction and significant injury-induced neointimal hyperplasia in the injured carotid artery 4 weeks post-surgery. Treatment with resistin further decreased the lumen diameter compared to the saline-treated mice (Fig. 4A and Supplementary Fig. 6). Resistin-mediated luminal-narrowing was prevented by continuous subcutaneous εV1-2 treatment (Fig. 4A). Change in lumen diameter in mice treated with εV1-2 alone was not different compared to saline treated (control) mice. Plasma analyses from the mice showed significant increase in systemic ROS levels in resistin-treated mice compared to saline controls (Supplementary Fig. 7A and B), and there was no difference between saline treated and εV1-2/resistin-treated mice. Analyses of plasma showed 4.5 fold higher resistin levels in mice implanted with resistin pump. Elevated levels of tissue ROS were also observed in the carotid-artery tissue samples isolated from mice treated with resistin compared to the saline control (Fig. 4B and Supplementary Fig. 7C). There was no significant increase in carotid tissue ROS in the resistin/εV1-2 group compared to the control. Mice treated with εV1-2 treated alone did not show any increase in systemic or tissue ROS. Based on our *in vitro* results we tested Nox activity in the injured carotid artery in the 4 groups of animals. As shown in Fig. 4C, resistin infusion increases Nox activity by ~1.6 fold and this is inhibited by PKCε inhibitor. Additionally, superoxide derived ROS in the injured arteries was confirmed in the presence of catalase as shown in Supplementary Fig. 8.

Discussion

To our knowledge this is the first comprehensive study to evaluate the roles that resistin-mediated ROS generation plays in atherosclerosis, in the context of molecular interaction, cellular effects, and *in vivo* influence. We show that systemic resistin correlates to circulating oxidative stress in patients with severe atherosclerosis, induces VSMC dysfunction *in vitro*, and exaggerates intimal hyperplasia *in vivo*. Mechanistically, we observe that resistin promotes dose and time-dependent ROS generation and acute-phase inflammatory cytokine production in VSMCs, and that PKCε mediates resistin-induced cellular and molecular effects. Both chronic and acute effects of resistin contribute to the novel mechanistic basis for resistin-associated cardiovascular effects.

Resistin has been shown to act on a variety of cell types including VSMCs.[18–20,38,39] However, research to date primarily focused on the phenotypical properties of VSMCs. We also observed phenotypical changes in VSMCs following resistin treatment, implying its passive role in atherosclerosis. We saw, for the first time, that resistin also stimulates VSMCs to release inflammatory cytokines, a function normally attributed only to inflammatory cells. In addition, we demonstrated that PKCε-mediated Nox activation also governs resistin-induced inflammation in VSMCs and subsequent VSMC dysfunction, suggesting a direct role of resistin on VSMCs, and an active role of VSMC in inflammatory process. Both TNFα and IL-1β are considered potent stimuli of the acute-phase reaction and important activators of lymphocytes, proposing that VSMCs play a dynamic role in atherosclerosis.[40–42]

Chronic inflammatory cascades in atherosclerosis[43] have been shown to be mediated by oxidative stress. Only one previous study by Gan et al. has shown that resistin induces ROS generation via Nox activation in VSMCs in a monocyte-SMC co-culture system.[24] Consistently, we too observed that resistin significantly triggered ROS generation. In addition, we further demonstrated a time-dependent and dose-dependent increase in ROS following resistin treatment. Unlike Gan *et al.,* we observed Nox4 being the primary isoform activated by resistin after 2 hours of treatment as opposed to Nox1. It is possible that Nox 4 is activated early whereas Nox 1 activation follows later and happens only in the presence of monocytes or in extremely high concentration of resistin, such as at 100ng/mL used by Gan et al. Our observations of sustained ROS levels suggest involvement of other ROS species in addition to the superoxide generated Nox.

Furthermore, in this study we identified an upstream regulator PKCε, suggesting that ROS production is inherently coupled to PKCε activation, thereby regulating pathways associated with VSMC dysfunction. Time course studies reveal that PKCε activation occurs as early as 5 minutes of resistin treatment while cytosolic ROS generation is seen 30 minutes following resistin treatment, confirming that this member of the novel PKC family is indeed a master regulator. Receptor-mediated action has been reported for resistin and TLR4 has been reported by several groups.[44,45] Crosstalk between TLR4 and Nox has also been shown in different cell types including macrophages, monocytes, and VSMCs.[46,47] Nox activation, however, is not restricted only to TLR4.[48] We speculate that TLR4 may likely be a receptor for resistin in VSMCs. Our preliminary observations suggest such interaction, though further investigations are needed.

Nox activation produces superoxide, which is usually the first stage in the ROS-forming cascade, producing other species of ROS thereafter. Superoxide dismutase activity was however not affected by resistin. It is still possible that hydrogen peroxide, formed from Nox generated superoxide, can produce highly reactive radicals leading to sustained ROS. Based on earlier studies we speculate that the possible downstream targets are MMP9 (in migration) and Erk and Akt (proliferation). Here, we observed that inhibition of Nox, downstream of PKCε, blocked VSMC migration as well as MMP activation (Supplementary Fig. 9). This observation is consistent with our previous findings in PKCε inhibition.[21] Similarly, quenching ROS inhibits resistin-induced proliferation via Erk and Akt signaling pathways (data not shown). Besides, ROS may regulate additional signaling pathways such as JNK and NF-κB, which also govern inflammation and cell survival.[49] Taken together, these results suggest that VSMC dysfunction mediated by resistin is involved in the onset of pathological states and is primarily regulated by oxidative stress.

An earlier study reported that neointimal hyperplasia in rats exposed to balloon injury was mediated by resistin-induced oxidative stress.[50] Our study differs in that it is conducted in a transgenic murine model i.e. *ApoE*$^{-/-}$, known to develop advanced atherosclerotic lesions resembling humans, and that the model is consistent with a clinical scenario in which in-line blood flow is maintained during and after vessel injury. In addition, our findings on the role of PKCε in resistin-induced oxidative stress and intimal hyperplasia are novel. While *ApoE*$^{-/-}$ mice are known to be atherogenic and the effectiveness of guide-wire injury has been demonstrated in our previous study,[34] we observed exaggerated intimal hyperplasia

after systemic resistin treatment regulated by PKCε, possibly via Nox as evidenced by the upregulation of Nox activity in the injured carotid artery (Fig. 4C). At this juncture, we cannot rule out the effects of resistin on other functions. More focused studies involving a Nox isoform gene knock-out mice or RNA silencing studies need to be performed and are beyond the scope of this investigation.

In conclusion, this study reveals novel molecular links between PKCε, ROS, and inflammation within vascular smooth muscle cells that are relevant in the pathogenesis of atherosclerosis. As an upstream modulator, PKCε may be a potentially novel therapeutic target in resistin-associated poor cardiovascular outcomes, and this new therapeutic option have the potential to translate into reduced morbidities and mortality for elderly patients with atherosclerotic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VSMC	vascular smooth muscle cell
PKCε	protein kinase C epsilon
ROS	reactive oxygen species
Nox	NADPH oxidase
***ApoE*$^{-/-}$**	apolipoprotein E knockout
CVD	cardiovascular disease

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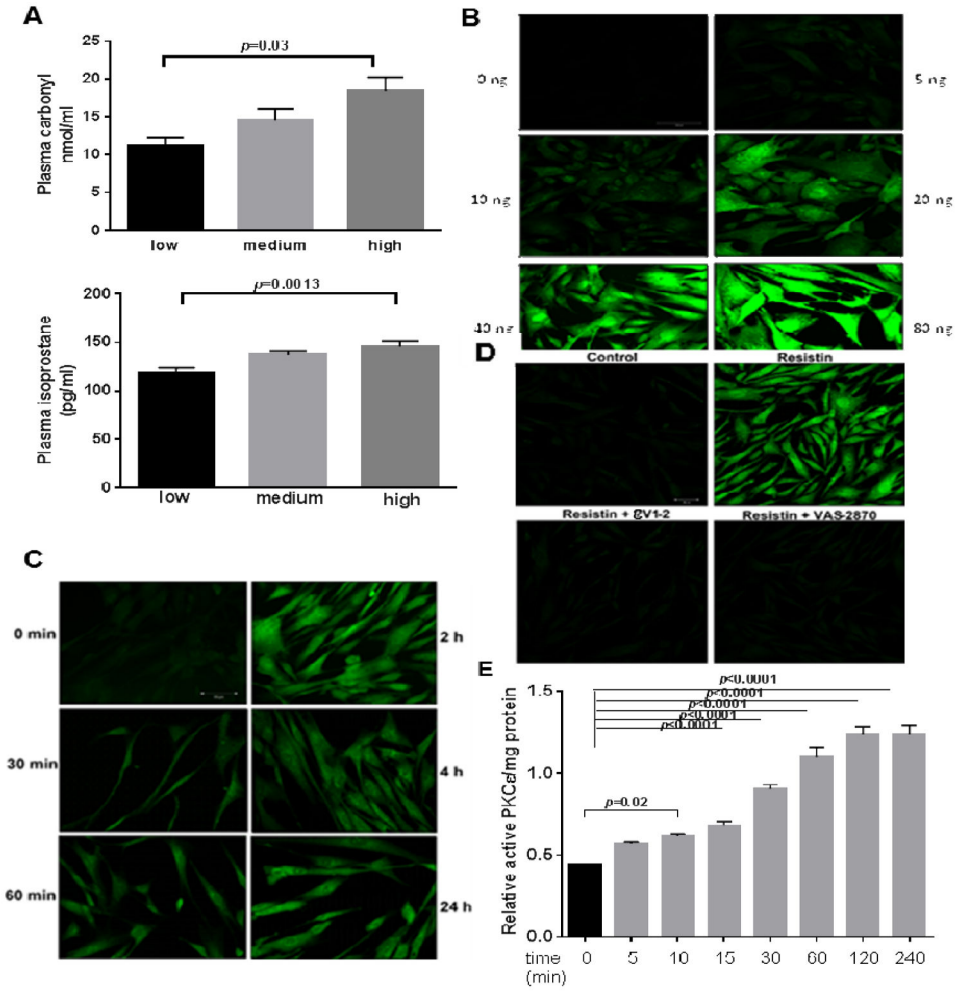
highlights

- Higher resistin levels are associated with increased systemic oxidative stress in humans undergoing cardiovascular interventions
- Resistin induces ROS in vascular smooth muscle cells via PKCε mediated activation of NADPH oxidase
- NADPH oxidase 4 isoform is involved in the process confirmed using siRNA studies
- Resistin potentiates vascular smooth muscle cell dysfunction and inhibition of PKCε completely revokes the effect
- Resistin exacerbates carotid thickening in a *ApoE* knock-out mouse model of guide wire injury induced hyperplasia via PKCε



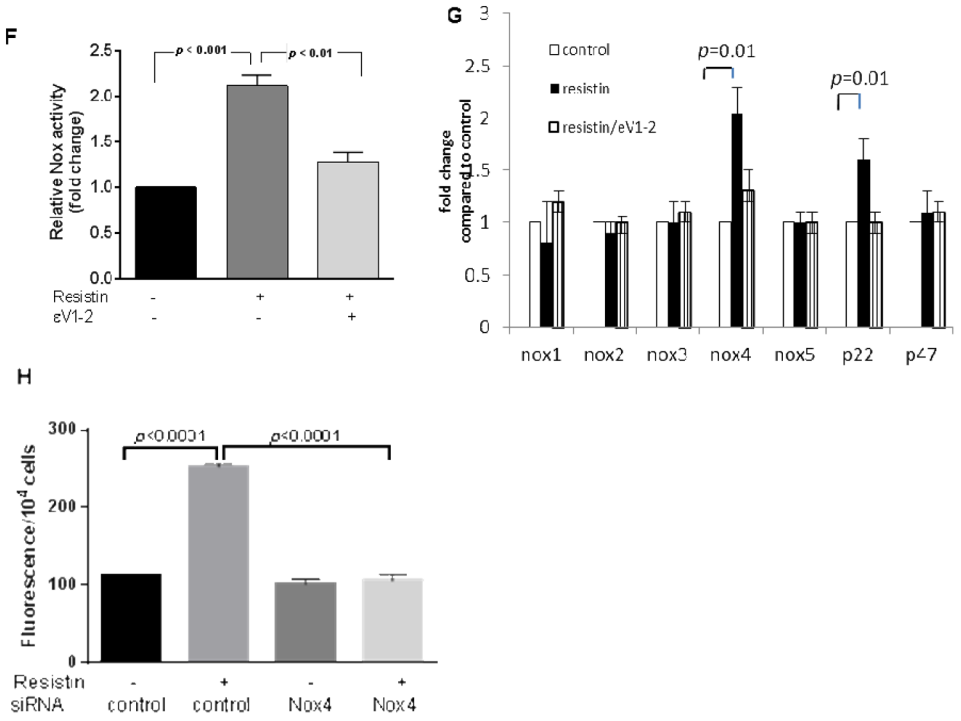


Fig. 1. Resistin is associated with ROS in human plasma, and it activates specific Nox isoforms in VSMCs via PKCε

(A) Human plasma levels of resistin were associated to plasma carbonyls and isoprostane levels. (N=20) analyzed for protein carbonyl and isoprostane in plasma. Resistin levels corresponds to low (1–3.4ng/ml); medium (3.4–4.9 ng/ml) and high (4.9–13.3 ng/ml) respectively. VSMCs treated with different concentration of resistin for various time periods were analyzed for cytosolic ROS by (B and C) confocal microscopy. Effects of Nox inhibitor (VAS 2870; 10 μM) and PKCε inhibitor (eV1-2; 1 μM) on resistin-induced cytosolic ROS are shown in (D). Cells treated with 40ng/mL resistin and Nox activity shown as fold change based on control (E). (F) Change in expression of Nox subunits as analyzed by real time PCR is shown. Specific PKCε activity was measured (G). Effect of *Nox4* siRNA inhibition on resistin induced ROS is shown in (H). Data are shown as mean ± S.E.M of at least 4 independent experiments in duplicate. *p<0.05; ***p<0.0005; #p<0.0001 by 1-way or 2-way ANOVA. Values are representative of at least 4 independent experiments.

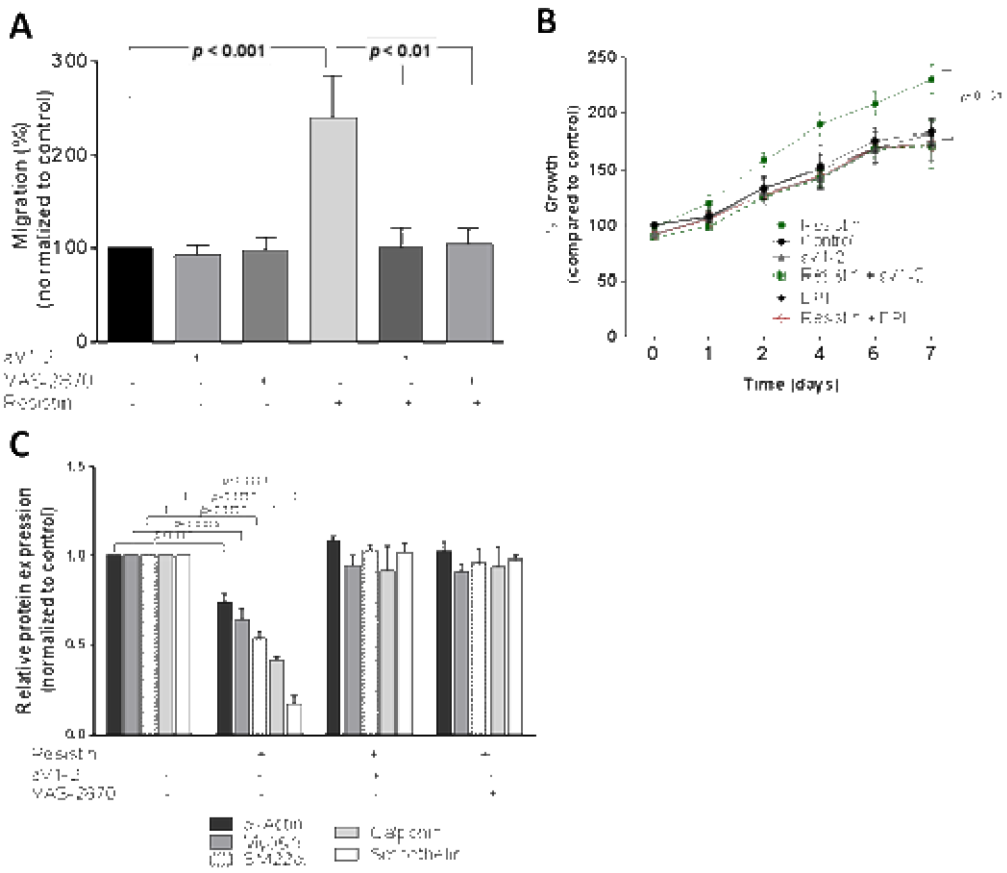


Fig. 2. Resistin affects VSMC function

VSMCs were treated with Nox inhibitor (DPI or VAS-2870) or PKCε inhibitor (εV1-2) in the presence or absence of resistin and analyzed for migration (A), proliferation using growth curves (B) and dedifferentiation by western blot quantification shown in (C). Data are shown as mean ± S.E.M of at least 4 independent experiments in duplicate analyzed by 1-way or 2-way ANOVA. Values are representative of at least 4 independent experiments.

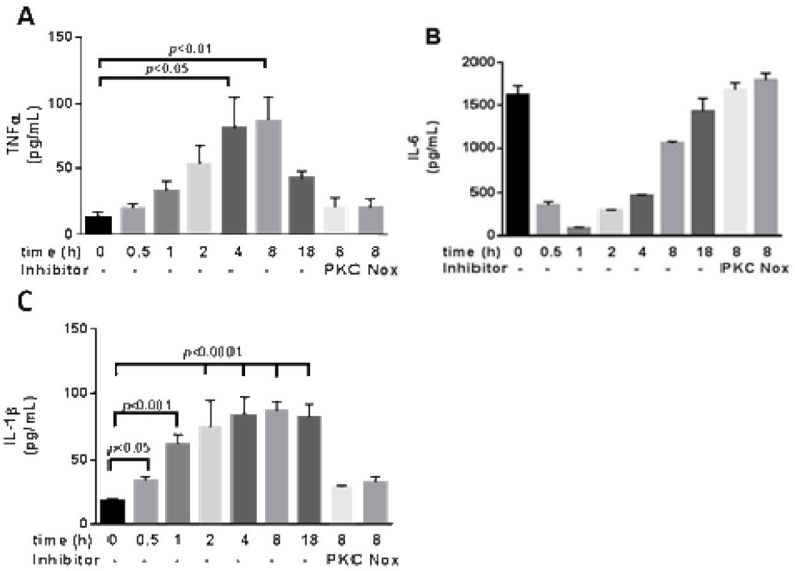


Fig. 3. Resistin induces inflammatory cytokine secretion from VSMCs mitigated by PKCe and Nox

VSMCs (equal number) were treated with resistin in the absence or presence of εV1-2 or VAS-2870 or DPI for 8h and conditioned media collected was analyzed for TNFα, IL-6 and IL-1β as shown in (A–C). Data are shown as mean ± S.E.M. analyzed by non-parametric Mann Whitney test

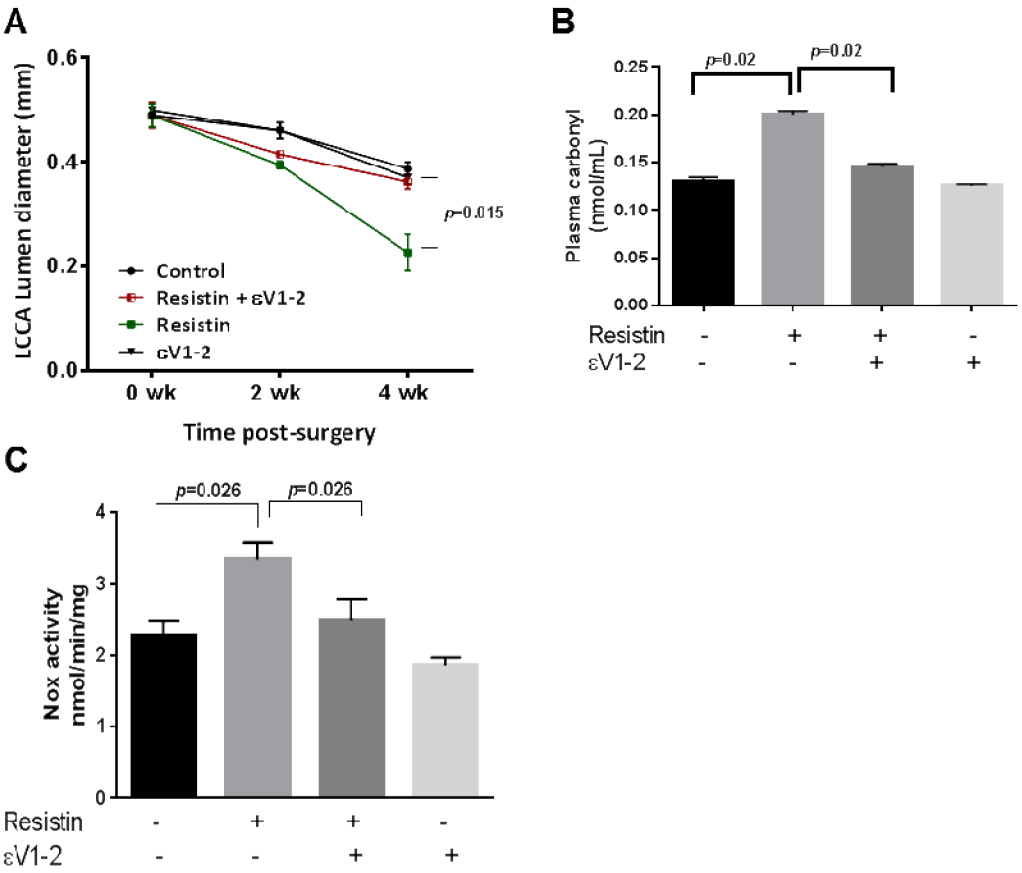


Fig. 4. Resistin exacerbates guidewire injury induced intimal hyperplasia in *ApoE*$^{-/-}$ mice

4 groups of *ApoE*$^{-/-}$ mice treated either with saline, resistin, resistin/εV1-2 and εV1-2 underwent wire injury in the left carotid artery. Ultrasound imaging was performed to measure the narrowing of the artery every other week post-surgery with at least 6 to 8 mice in each group. The luminal diameter of the left carotid artery is shown in (A). Oxidative stress (B) and Nox activity (C) in tissue as shown. Data are shown as mean ± S.E.M by non-parametric Mann Whitney test.

Table 1

Primers for real time PCR.

Gene	Forward	Reverse	GenBank No.
NOX1	5′-TTCACCAATTCCCAGGATTG AAGTGGATGGTC-3′	5′-GACCTGTCACGATGTCAGT GGCCTTGTCAA-3′	AF127763
GP91phox	5′-GTCACACCCTTCGCATCCA TTCTCAAGTCAGT-3′	5′-CTGAGACTCATCCCAGCCAGTG-3′	NM_000397
NOX3	5′-ATGAACACCTCTGGGGT CAGCTGA-3′	5′-GGATCGGAGTCACTCCC TTCGCTG-3′	AF190122
NOX4	5′-CTCGAGGAGCTGGCTCGC CAACGAAG-3′	5′-GTGATCATGAGGAATAGCA CCACCACCATGCAG-3′	AF261943
P22phox	5′-AACGAGCAGGCGCTGGCGT CCG-3′	5′-GCTTGGGCTCGATGGGCGTC CACT-3′	NM_000101
P47phox	5′-AGTCCTGACGAGACGGAAGA-3′	5′-GGACGGAAAGTAGCCTGTGA-3′	NM_000265
P67phox	5′-GGAGTGTGTCTGGAAGCAG-3′	5′-AGTGTGTAGGGCATGGAAC-3′	NM_000433
18SrRNA	5′-GTAACCCGTTGAACCCCATT-3′	5′-CCATCCAATCGGTAGTAGCG-3′	M10098
SM22α	5′-AACAGCCTGTACCCTGATGG -3′	5′-CGGTAGTGCCCATCATTCTT -3′	NP_003177.2
SM-MHC	5′-GCTGGAAGACACACTGGACA-3″	5′-CCAGGTCTGCGTTCTCTTTC -3	AAI43365.1
α-ACTIN	5′-AGGTAACGAGTCAGAGCTTTGGC-3′	5′-CTCTCTGTCCACCTTCCAGCAG–3′	X13839.1
SMOOTHELIN	5′-TTGGACAAGATGCTGGATCA -3′	5′-CGCTGGTCTCTCTTCCTTTG-3′	NM_198501.2
CALPONIN	5′-CATGACGGTGTATGGGCTGCCA-3′	5′-TAGGCGG AATTGTAGTAGTTGT-3′	BC138864.1
GAPDH	5′-TGCACCACCAACTGCTTAGC-3′	5′GGCATGGACTGTGGTCATGAG-3′	GI:182976