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Pathogenic role of angiotensin II and oxidized LDL in obstructive sleep apnea

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ABSTRACT: A sustained elevation of oxidative stress in patients with obstructive sleep apnea syndrome (OSAS) might help to explain their increased risk for cardiovascular diseases. We tested the hypothesis that the values of oxidative stress are increased in otherwise healthy subjects with OSAS when compared to closely matched control subjects.

We performed a prospective study of 38 subjects who did not have OSAS, and 37 patients with OSAS. Plasma indices of Ang II, vascular endothelial growth factor (VEGF), oxidized LDL (oxLDL), and circulating endothelial precursor cells (CEPs) were measured in OSAS patients and in matched controls. Peripheral blood mononuclear cells (PBMCs) were obtained from both groups, and then cocultured with endothelial cells to examine the effects on tube formation.

The OSAS group showed increased levels of Ang II, VEGF, oxLDL, and CEPs, which were decreased after nasal continuous positive airway pressure (nCPAP) treatment. *In vitro*, PBMCs from the OSAS group induced tube formation. Ang II, oxLDL, and Ang II-stimulated PBMCs induced lectin-like oxidized LDL receptor expression and VEGF receptor-2 activation on endothelial cells, respectively.

These observations suggest an important role of Ang II and oxLDL-mediated LOX-1 upregulation in endothelial cell injury in patients with OSAS.

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Obstructive sleep apnea syndrome (OSAS) is a highly prevalent disorder affecting approximately 4% of adults (1). The predominant physical morbidity of the disorder is cardiovascular, and OSAS is an independent risk factor for cardiovascular diseases, in particular systemic arterial hypertension (2, 3) but also coronary artery disease, congestive cardiac failure, and cerebrovascular events (4). In patients with OSAS, oxygen saturation may repeatedly decrease during the apneic events. A prominent physiologic adaptive response of tissue to hypoxia or ischemia is angiogenesis, the formation of new blood vessels and increasing the blood supply (5). Vascular endothelial growth factor (VEGF) regulates multiple endothelial cell functions including mitogenesis, vascular permeability, and vascular tone (6, 7). VEGF has been shown to be upregulated by angiotensin II (Ang II), which promotes ischemia-induced angiogenesis *in vitro* and *in vivo* (8, 9). Numerous reports have shown that VEGF expression is significantly increased in the plasma of OSAS patients (10-14) and is induced by Ang II in peripheral blood mononuclear cells (PBMCs) (10). Furthermore, Ang II upregulates the expression of LOX-1, a lectin-like scavenger receptor for oxidized low-density lipoprotein (oxLDL) in endothelial cells (15). In turn, the activation of LOX-1 also upregulates the Angiotensin II receptor (16). This positive feedback loop between LOX-1 and Ang II receptor activation increases Ang II-induced angiogenesis. These angiogenic factors are known to potentiate the proliferation and recruitment of bone-marrow-derived circulating endothelial progenitor cells (CEPs) from bone marrow (15). Recent studies have provided increasing evidence that the formation of new blood vessels in postnatal life does not result solely from the sprouting of pre-existing vessels, but also involves the recruitment of bone-marrow-derived progenitors for endothelial cells (16). Postnatal vasculogenesis mainly contributes to vascular healing in response to vascular injury or

ischemia through the processes of rapid endothelialization of denuded vessels and collateral vessel formation (16). In this process, CEPs home to the site of injury and work in concert with existing mature endothelial cells (16). It is generally agreed that VEGF is increased in the patients with OSAS, but vasculogenesis and the role of CEPs in OSAS is unclear (17-19). Recurrent intermittent hypoxemia in OSAS may induce the generation of an excess of reactive oxygen species (ROS), which can ultimately result in endothelial cellular injury *via* reactions with proteins, nucleic acids, and lipids. Whether there is indeed increased oxidative stress in OSAS is controversial (20-24). These conflicting results may be explained by several factors including the presence of co-morbidities and medications in OSAS patients, both of which can have significant effects on measurements of oxidative stress. OSAS may be a hallmark of CEPs' mobilization and oxidative stress.

To more easily recognize the impact of these factors, more research must be done so as that their effects and the roles that they play in obtaining results can be better understood. Therefore it was the purpose of this study to test the hypothesis that the plasma level of angiogenic factors, CEPs, and the lipid peroxidation are increased in otherwise healthy subjects with OSAS when compared with closely matched control subjects proved to be free of any other diseases. By proving this hypothesis, we can show a part of the mechanism that underlies OSAS that will help us to better understand the many factors that contribute to this ailment.

MATERIALS and METHODS

Patients and control subjects

Males with suspected OSAS, who had sleep-related symptoms (i.e., snoring, witnessed apneas, excessive daytime sleepiness) and no other medical disorders, were considered for the study, which was approved by the Iwate Medical University Hospital Ethics Committee from the patients who sought treatment within their facilities. Prospective patients were then notified of our desire to include them in our study and if they would be willing to participate. Upon acceptance, the subjects were provided written informed consent according to the ethical protocols of our institution (see the online depository 1).

Measurement of Ang II, VEGF, and oxLDL

Blood samples were drawn from subjects' antecubital veins at the end of each sleep study (i.e., 6:00 AM). Further procedures are given in the online depository 2.

For quantification of CEPs

We studied the peripheral blood level of CEPs in 8 of the most severe OSAS patients whose apnea-hypopnea index (AHI) values were greater than 60, and eight age- and BMI-matched control subjects (see the online depository 3).

Reverse transcriptase-polymerase chain reaction (RT-PCR) for mRNA expression and immunoblotting analysis of lectin-like oxLDL receptor (*LOX-1) on human aortic endothelial cells (HAECs)*

HAECs were purchased from Kurabo (Osaka, Japan) and maintained in a Humedia-EG2 medium (Kurabo) according to the manufacturer's instructions. Stimulated HAECs were submitted to RT-PCR and immunoblotting (25) (also see the online depository 4).

For coculture of PBMCs treatment with human umbilical vein endothelial cells (HUVECs) or HAECs

In the coculture test, we used PBMCs obtained from 8 sever OSAS patients and 8 age- and BMI-matched control subjects as mentioned above. Heparinized venous blood was centrifuged on Histopaque 1077 (Sigma, Tokyo, Japan) at 600x g for 20 min at room temperature. The detail protocol of PBMCs' isolation has been reported elsewhere (10) (also see the online depository 5). Coculture experiments were performed in 24-well plate ThinCert Cell Culture Inserts systems (membranes with pore size of 0.4 μm in diameter, growth area of membrane 33.6 mm^2, Invitrogen Corp.) with HAECs or HUVECs plated at the bottom of the wells. PBMCs ($1x10^6$/ ml or $1x10^4$/ ml) were seeded onto the inserts, and thereafter incubated overnight at 37 ^oC, 5% CO_2 in air for the indicated times.

Modified in-vitro coculture angiogenesis

In vitro angiogenesis was assessed by the formation of capillary-like structures of HUVECs cocultured with human diploid fibroblasts (see the online depository 6). The experimental procedure followed the instructions provided with the Angiogenesis kit (Kurabo). On day 3, PBMCs were collected and RNA was extracted from PBMCs (see the online depository 7). The new PBMCs from another patient were seeded onto the inserts in the new medium, starved for 24 h, and then followed by repeated stimulation and the preincubation as mentioned above. This procedure was repeated on days 5, 7, 9, 11, 13, and 15. On day 17 HUVECs were submitted to counter staining (Figure 1, also see the online depository 8).

Coculture for LOX-1 expression and VEGF receptor-2 (VEGFR-2) phosphorylation on HAECs

HAECs ($1x10^5$) were plated 72 h prior to assay in 24-well plates so they would be 50 to 60% confluent when cocultured with PBMCs, which were seeded onto inserts on day 1. The replacement of PBMCs in the inserts and medium, and the condition of incubation with Ang II, valsartan, or soluble VEGFR-2 were as described in the procedure of *in vitro* angiogenesis. On day 17, HAECs were submitted to immunoblotting to detect LOX-1 protein expression (see the online depository 4) or examine VEGFR-2 phosphorylation using the in-cell western blotting method (26) (Figure 1, also see the online depository 9).

Statistical analysis

All data are expressed as the mean± SEM. The one-way ANOVA and Post hoc tests were used to assess the statistical significance among the groups. The student unpaired *t* test was also used to examine changes between the control group and the OSAS patients after testing data for the normality of the population using the Kolmogorov-Smirnov test.

RESULTS

Patient and control population

Of the 77 subjects initially examined, 39 patients had OSA. Since two of the subjects were newly diagnosed asthma and allergic rhinitis after the study, the non-OSA control and the OSAS group consisted of 38 and 37 individuals, respectively. The non-OSAS control individuals were informed of the clinical features of OSAS including significant excessive daytime sleepiness and snoring but demonstrated no objective finding of sleep-disordered breathing in polysomnography (PSG) studies. The baseline

characteristics of the study population are described in Table 1. The two groups were similar in age and moderate obesity. AHI and oximetry data were significantly different between the two groups ($p< 0.01$). No subject had clinical evidence of any other medical disorder, and each subject's detailed biochemical profile, including liver and renal function, cardiac enzymes, and resting electrocardiogram, were within normal limits. Of the original 37 OSAS patients recruited, only the 8 worst OSAS patients were used for this *in vitro* study because they had an AHI>60, therefore, for the control patients with similar age and BMI were used.

Measures of Ang II, VEGF, and oxLDL

Table 2 shows the levels of oxLDL in the present study. Twenty nine suitable subjects with OSAS, who had agreed to possible continuous positive airway pressure (CPAP) therapy before undergoing diagnostic sleep study, were considered. Eight subjects dropped out due to intolerance of CPAP. The remaining 29 subjects underwent repeated sleep studies after 12 weeks of therapy. All were evaluated for symptoms and side effects, and objective compliance data was downloaded from the devices. In patients with OSAS, the levels of Ang II, VEGF, and oxLDL were significantly higher than in the control and post-CPAP group (Table 2; *, $p<0.05$ vs. Control and post-CPAP, Ang II and VEGF data not shown). After 12 weeks of CPAP therapy in the 29 CPAP-treated subjects with OSAS, all three data points decreased (Table 2; *, $p<0.05$ vs. Control and post-CPAP, Ang II and VEGF data not shown), but there was no noticeable decrease in other observed parameters.

The numbers of bone-marrow-derived CEPs

Hypoxia in the night and high concentrations of Ang II and VEGF that were found in the circulation of patients with OSAS strongly suggested that CEPs and their stem cells in the bone marrow responded to these angiogenic factors. White blood cells from 8 of the most severe OSAS patients (AHI>60) and controls were used for this experiment and the subsequent experiments of cocultivation with ECs. Flow-cytometric analyses for the staining of CD133, CD34, and CD202b (Tie-2) on the gated CD45-negative cells of the participants are shown in Table 2. Individuals with OSAS had a ~3-fold increase in $CD133^{+}CD34^{+}CD202b^{+}CD45^{-}$ cells in their blood circulation compared with the control group. After continuous positive airway pressure (CPAP) treatment, this increase was suppressed (Table 2; *, $p<0.05$ vs. Control and post-CPAP).

Influence of Ang II and oxLDL on endothelial receptor for oxLDL (LOX-1) expression in HAECs

Having shown the increased plasma levels of Ang II, VEGF, and oxLDL in the OSAS patients, we next investigated the interaction between these molecules and HAECs *in vitro*. We evaluated whether oxLDL and Ang II induce LOX-1 expression on HAECs.

As shown in Figure 2, a low concentration of oxLDL led to the expression of LOX-1. The maximum LOX-1 expression occurred in response to 10 μg /mL concentration of oxLDL (Figure 2; *, $p<0.05$ vs. 0 μg/ml). In contrast, we observed that LOX-1 expression increased in response to Ang II in a concentration-dependent fashion (Figure 3; *, $p<0.05$ vs. 0 nM). Based on these findings, we chose the maximum effective concentrations of oxLDL (10 μg /mL) and Ang II (100 nM) for the subsequent experiments. When HAECs were stimulated with oxLDL-1 and Ang II, the level (mRNA and protein) of LOX-1 was additive for both stimuli (Figure 4; *, $p<0.05$ vs. no treatment).

PBMCs from OSAS patients induce tube formation via VEGFR-2 phosphorylation in the modified coculture system

The increase of LOX-1 expression by Ang II led us to determine whether this molecule affects another known response of ECs, angiogenesis. Since Ang II has been reported to be able to induce VEGF from PBMCs (10), we thought it possible that the tube formation is dependent on VEGF which may be secreted from PBMCs. To test this idea, we put inserts in the angiogenesis kit and seeded PBMCs onto the inserts to detect their interaction with HUVECs (Figure 1). As shown in Figure 5A upper panel and 5B, the tube formation by cocultivation with PBMCs was significantly increased when the PBMCs were exposed to Ang II *in vivo* and were obtained from OSAS patients, while little tube formation was observed when the cells were exposed to medium only. This effect of PBMCs obtained from OSAS patients was absent in the presence of sVEGFR-2. Similar results in the mRNA level of $VEGF_{165}$ were observed when PBMCs were subjected to RT-PCR (data not shown). Of note, sVEGFR-2 did not interfere with $VEGF_{165}$ mRNA expression in PBMCs. These results suggest that VEGF from OSAS PBMCs contributes to tube formation. As PBMCs from patients with OSAS expressed VEGF, cultured PBMCs from normal subjects were further analyzed in tubular formation and phosphorylation of ECs experiments after treatment with Ang II. As shown in Figure 6A upper panel and 6B, the tube formation by PBMCs from controls was significantly increased after stimulation with Ang II. The biological effects of Ang II are mediated by the activation of Ang II receptors, of which 2 major subtypes, AT1R and AT2R, have been identified. Most of the cardiovascular actions of Ang II have been attributed to AT1R activation. Pretreatment with AT1R blocker valsartan and sVEGFR-2 markedly

suppressed the tube formation induced by PBMCs from control subjects with Ang II stimulation. The inhibition of tube formation by these factors was not due to any expected toxicity, because nonspecific IgG could not inhibit tubular formation. These results clearly indicated that the tube formation induced by PBMCs from controls was highly dependent on VEGF secretion. Having identified Ang II induced-VEGF in PBMCs as an inducer of EC tube formation, we next sought to confirm the activation of VEGFR-2 on HAECs. It has previously been reported that tyrosine residues on VEGFR-2 are autophosphorylated in response to VEGF, and that the phosphorylation of VEGFR-2 at Tyr^{1214} has a central role in the activation of this receptor. PBMCs from OSAS led to the dose-dependent phosphorylation of VEGFR-2 at Tyr^{1214} ($p<0.05$; Figure 5A lower panel and 5C). Soluble VEGFR-2 strongly inhibited the stimulatory effect of the phosphorylation of VEGFR-2 on HAECs (Figure 5A lower panel and 5C). In keeping with our tubular formation data, the increased receptor phosphorylation occurred in response to cocultivation with Ang II-stimulated PBMCs or VEGF stimulation (Figure 6A lower panel and 6C; $p<0.05$). Pretreatment with valsartan and sVEGFR-2, but not nonspecific IgG, markedly suppressed the VEGFR-2 phosphorylation by Ang II-stimulated PBMCs. These increased tubular- formed endothelial cells expressed LOX-1 protein (Figure 6A middle panel). Thus, we concluded that Ang II induced VEGF *via* the AT1R on PBMCs, resulting in an increase in tube formation and the expression of LOX-1 protein.

DISCUSSION

Several new findings have emanated from this study. First, we have demonstrated enhanced angiogenesis, vasculogenesis, and lipid peroxidation, oxLDL in OSAS patients

when compared with controls, which was reversed by CPAP therapy. Second, we have shown that oxLDL and Ang II induced LOX-1 expression on endothelial cells. Finally, we have shown that Ang II induced ECs tube formation, which express LOX-1, *via* PBMCs.

Our *in vivo* study has a number of strengths compared with some previous studies. We took care in selecting subjects to exclude smokers or anyone with a cardiovascular or other medical disorders, or who was taking any medication. Furthermore, we excluded subjects with sunburn, because even physiological doses of ultraviolet A radiation administered at above 300 W/m^2 have previously been shown to induce oxidative damage to proteins in human skin fibroblasts (27). Thus, the study population allowed the evaluation of relationships between ROS and OSAS without the potential influence of confounding factors.

The role of inflammation in the development of atherosclerosis is well established. Once activated by various stimuli (e.g., low-density lipoprotein cholesterol, injury, or infection), ROS oxidize lipids, injure cell membranes, create a proinflammatory milieu, and denature the potent vasodilator species nitric oxide (28, 29). LDL is generally believed to be important in the development of atherosclerosis, and its atherogenicity may be due to oxidative modifications, oxLDL (30, 31). Because LDL is oxidized under the influence of super oxide and incorporated into macrophages, thus forming foam cells, we propose that repetitive hypoxia / reoxygenation could facilitate free radical production. Also, Ang II was shown to be a potent stimulus for ROS generation (32), which was increased in patients with OSAS when compared with normal control subjects in this study. Our findings are in agreement with Møller and colleagues (33). They have shown that untreated OSAS is associated with an upregulation of the systemic

angiotensin-aldosterone system, and that amelioration of the nocturnal hypoxemia by long-term CPAP reduces the blood pressure (BP). Interestingly, this reduction in BP was strongly correlated with a reduction in the plasma renin and Ang II concentration. Works from the Mehta laboratory have shown that oxLDL and Ang II decrease nitric oxide generation and increase lipid peroxidation and LDH release in cultured human coronary artery endothelial cells (34, 35). Thus, it is likely that Ang II and oxLDL are critical factors in atherogenesis. CEPs may play a role in endothelium maintenance, being implicated in both reendothelialization and neovascularization. Recent studies show that CEPs are incorporated into new and existing blood vessels in many pathological conditions, including tumor vascularization and myocardial ischemia, and that CEPs are elevated and serve as biomarkers for inflammatory diseases such as atherosclerosis (36) and cerebrovascular disease (37). Indeed, it has been demonstrated that the number of CEPs in the endothelium and in the intima correlated with the magnitude of the presence of hypoxia in patients with chronic obstructive pulmonary disease (38). Werner et al. have demonstrated that decreased endothelial progenitor cells in patients with coronary artery disease predicts the increased occurrence of cardiovascular events (39). They have also shown that using mouse model CEPs also contribute to vascular lesion formation by inducing smooth muscle cells proliferation and neointimal formation at sites of vascular injury (40). Accordingly, CEPs might exert opposite effects on injured vessels. An imbalance between growth factors, facilitated by the disruption of endothelial function and oxidative stress generated by intermittent hypoxemia, could determine the fate of CEPs. A few groups have reported a decrease or no differences in the number of CEPs between controls and patients with OSAS (17-19). One possible reason for this discrepancy may be due to the differences in studied populations and methods. Their

population of control and patients with OSAS included female subjects. Since several reports have demonstrated that the influence of the menstrual cycle affects on the number and the function of CEPs (41, 42), we recruited only male patients and excluded the possibility of the cyclical mobilization of CEPs. Moreover, since it is also recommended that using CD133, vascular endothelial cell marker, and CD34 in combination with a viability marker for quantification of EPCs in the blood (43), we used these three antibodies and CD45 to detect CEPs. In our context, VEGF and CEPs are also increased in OSAS patients compared to healthy controls, supporting the concept of a reendothelialized and neovascularized environment in the OSAS patients. Since there was a CPAP intervention in our study, which has provided further evidence of a link between OSAS and endothelial dysfunction by demonstrating that treatment of apnea-related hypoxia reduces oxidative stress and angiogenic factors, our present data *in vivo* supports the notion that oxidative stress, proarteriosclerosis and defective reendothelialization might help to characterize patients with OSAS.

Given this clinical data, *in vitro* experiments were directed at detecting the interaction of Ang II, oxLDL, PBMCs, and ECs. Many studies have shown that LOX-1 facilitates the uptake of oxLDL and mediates several of its biologic effects (44, 45). Kataoka et al. have shown that LOX-1 is upregulated in atherosclerotic tissues from humans (46). In the present study, the expressions of the LOX-1 was upregulated by oxLDL and Ang II (Figure 4). These observations may have important implications with regard to the propagation of endothelial cell injury in the presence of Ang II and oxLDL. Another study from our laboratory (10) showed that VEGF expression on PBMCs obtained from patients with OSAS is induced, at least in part, by increased Ang II. VEGF binds to two tyrosine kinase receptors on blood vessel endothelial cells, VEGFR-1/Flt-1 and

VEGFR-2/KDR/Flk-1. VEGFR-2 is the most biologically important receptor for VEGF in adults. It regulates endothelial cell migration, proliferation, and survival (47). Following its binding to VEGF, VEGFR-2 dimerizes and undergoes autophosphorylation on tyrosine residues within its cytoplasmic portion. We postulated that increased Ang II may enhance VEGF expression and tube formation via the phosphorylation of Tyr^{1214} within VEGFR-2. Indeed, our study demonstrated that Ang II-induced tube formation is dependent on VEGF secreted from PBMCs, and that phosphorylation of Tyr^{1214} within VEGFR-2 appears during endothelial cell tube formation in response to VEGF.

In conclusion, we demonstrated that the levels of Ang II, VEGF, oxLDL, and CEPs in peripheral blood in OSAS patients are increased. We also observed an increased angiogenesis when cocultured with PBMCs obtained from OSAS patients, which was associated with the expression of LOX-1. These findings suggest that recurrent intermittent hypoxemia may induce an ongoing process of endothelium repair and atherosclerosis. Ang II receptor blocker and nCPAP therapy may ultimately represent a potent strategy for clinical therapy of vascular diseases in patients with OSAS.

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Figure legends

Figure 1. Peripheral blood mononuclear cells (PBMCs) coculture experiments protocol. PBMCs obtained from 8 of the most severe OSAS patients or 8 age- and BMI-matched control subjects were used. PBMCs from one patient or one healthy control were plated onto the insert for 2 days. HAECs and HUVECs were exposed to angiotensin II (Ang II) plus Ang II-stimulated PBMCs for 17 days. Human umbilical vein endothelial cells (HUVECs) were subjected to immunocytochemistry (ICC), and human aortic endothelial cells (HAECs) were submitted to immunoblotting and in-cell western after the last PBMCs collecting. Pt, patient with obstructive sleep apnea; Con, healthy control subject; S, seeding and starvation; Co, collection of PBMCs and medium.

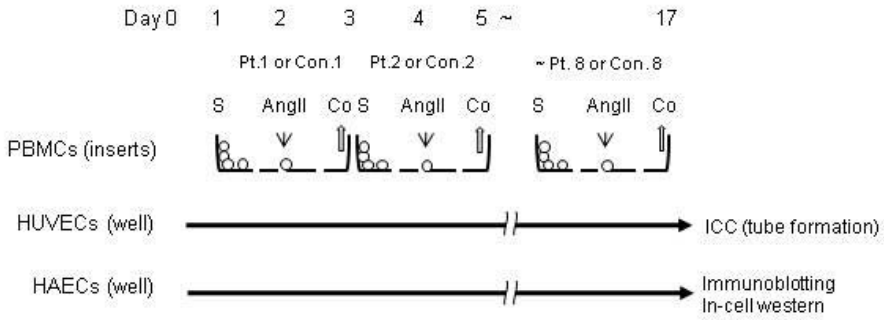

Figure 2. Endothelial receptor for lectin-like oxidized low- density lipoprotein (oxLDL) on human aortic endothelial cells (HAECs). The incubation of HAECs with oxLDL for 3

days upregulated the expression of lectin-like oxLDL receptor (LOX-1) mRNA. Angiotensin II (Ang II) at a concentration of 10μg/ml increased LOX-1 mRNA expression on HAECs. In contrast, 100μg/ml of Ang II induced less expression. LOX-1 protein band density was normalized by the housekeeping gene β-actin. The upper panel is representative of 5 separate experiments. The lower panel is the summary of the data (mean± SEM) from 5 experiments. *, $p<0.05$ vs. 0 μg/ml.

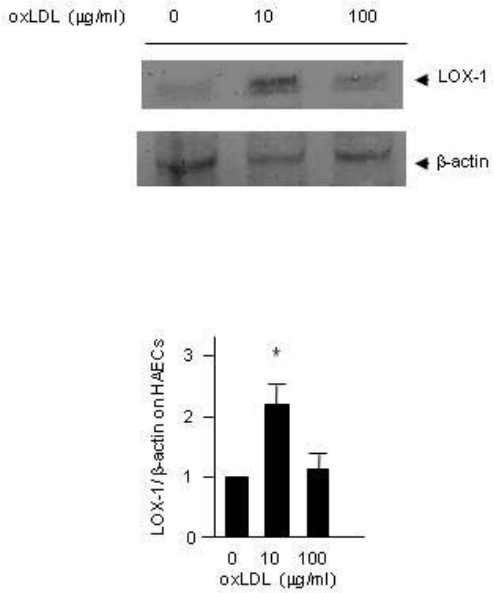

Figure 3. Lectin-like oxidized low-density lipoprotein (oxLDL) receptor (LOX-1) protein expression on human aortic endothelial cells (HAECs). Incubation of HAECs

with angiotensin II (Ang II) for 3 days increased the expression of LOX-1 mRNA in a concentration-dependent manner. The upper panel is representative of 5 separate experiments. The lower panel is the summary of the data (mean± SEM) from 5 experiments. *, $p<0.05$ vs. 0 nM.

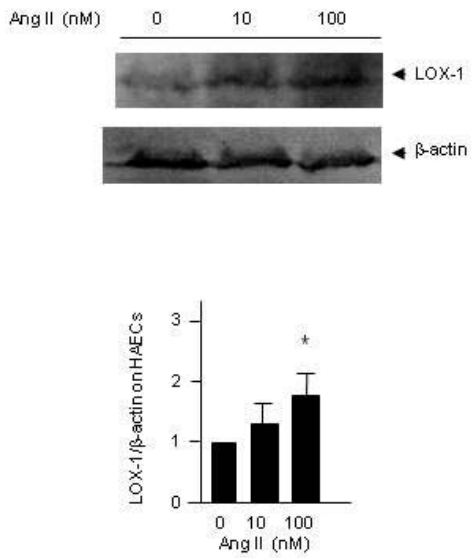

Figure 4. Upregulation of lectin-like oxidized low-density lipoprotein (oxLDL) receptor (LOX-1) mRNA and protein expression by oxLDL and angiotensin II (Ang II) in human

aortic endothelial cells (HAECs). Treatment with either oxLDL or Ang II caused a modest increase in LOX-1 expression in HAECs. The presence of both oxLDL and Ang II increased LOX-1 expression in a cumulative fashion. Left panel is representative of 5 separate experiments. Right panel is the summary of data (mean± SEM) from 5 experiments. *, $p<0.05$ vs. no treatment

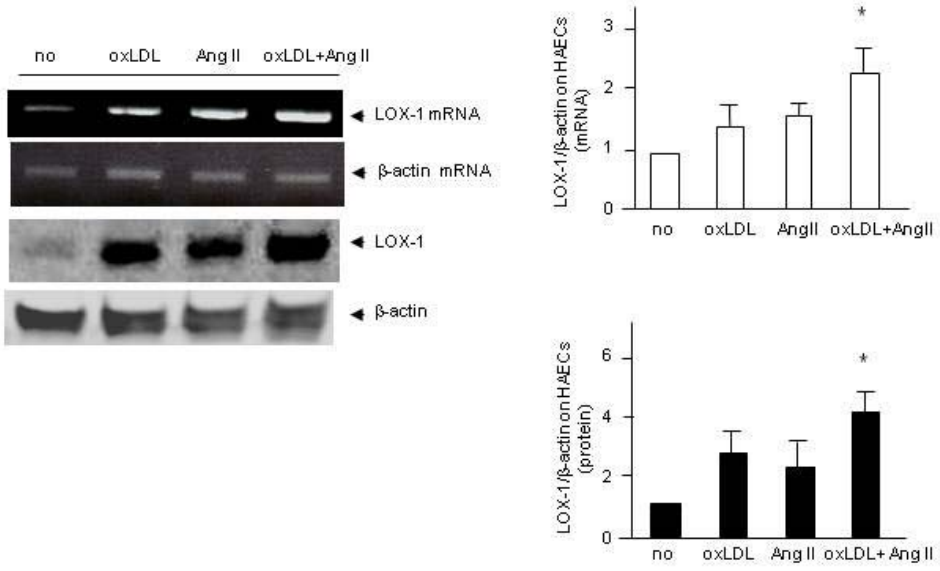
no
oxLDL
Ang II
oxLDL+Ang II
LOX-1 mRNA
β-actin mRNA
LOX-1
β-actin
LOX-1/β-actin on HAECs (mRNA)
0
1
2
3
*
no
oxLDL
AngII
oxLDL+AngII
LOX-1/β-actin on HAECs (protein)
0
2
4
6
*
no
oxLDL
Ang II
oxLDL+ Ang II

Figure 5. Peripheral blood mononuclear cells (PBMCs) from obstructive sleep apnea syndrome (OSAS) induce tube formation and the phosphorylation on Tyr^{1214} of vascular endothelial growth factor receptor-2 (VEGFR-2).

A, Human aortic endothelial cells (HAECs) were incubated with media alone (-) or cocultured with $1x10^6$ PBMCs from controls or $1x10^4$ and $1x10^6$ of PBMCs from OSAS patients for 17 days. PBMCs and medium were replaced with PBMCs from another patient in fresh medium every 3 days. At day 17, human umbilical vein endothelial cells (HUVECs) were fixed and submitted to immunostaining with anti-CD31 antibody for HUVECs to detect *in vitro* angiogenesis, which was assessed by the formation of capillary-like structures of HUVECs cocultured with PBMCs (top). On day 17, human aortic endothelial cells (HAECs) were also fixed and submitted to in-cell western blotting for VEGFR-2 and phosphorylated VEGFR-2 with a Li-Cor Odyssey system. The replacement of PBMCs in the inserts and medium was described in the procedure of the tube formation system. The image represents a 24-well two color in-cell western blotting with the 800 (green) and 700 (red) channels detecting total VEGFR-2 (as normalization) and phosphorylated VEGFR-2, respectively (bottom).

Lanes 1 and *2*, Endothelial cells were cultured only in medium (*Lane 1*, without anti VEGFR-2 antibody as a negative control); *Lane 3*, cocultured with PBMCs $1x10^6$ from control group; *Lane 4*, cocultured with PBMCs $1x10^4$ from OSAS; *Lane 5 and 6*, cocultured with PBMCs $1x10^6$ from OSAS (*Lane 6*, incubation with soluble form of VEGFR-2).

B, Ten different fields from the well were randomly selected, and tube formation was evaluated and quantified by the Chalkley counting method. PBMCs from OSAS increased the tube formation of HUVECs. The PBMC-mediated increased tube formation

was inhibited by the soluble form of VEGFR-2. Data are expressed as the mean ± SEM. *, P<0.05 compared with control.

C, In-cell western blotting showed the VEGFR-2 phosphorylation was low in HAECs cocultured with PBMCs from the control but increased markedly in HAECs cocultured with PBMCs derived from OSAS in a manner dependent on the number of PBMCs. This increase was suppressed by incubation with soluble VEGFR-2. Phosphorylated VEGFR-2 was normalized by the total VEGFR-2 expression. The fold increase in phosphorylated VEGFR-2 induced by PBMCs was calculated by comparison with the phosphorylated VEGFR-2 on HAECs cocultured with PBMCs from control. Data are expressed as the mean ± SEM. *, P<0.05 compared with control.

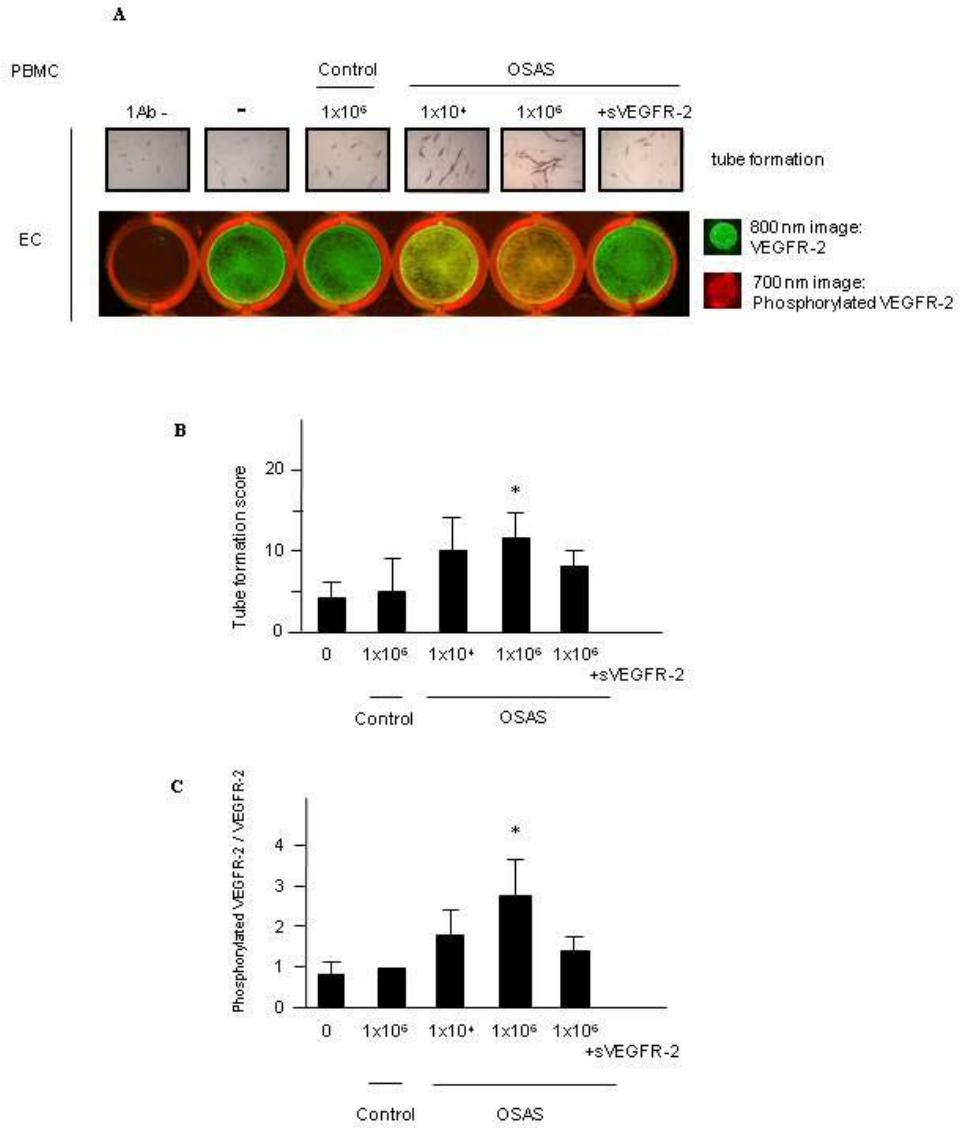

Figure 6. Angiotensin II (Ang II) and vascular endothelial growth factor (VEGF) from Ang II-stimulated peripheral blood mononuclear cells (PBMCs) induce tube formation,

expression of lectin-like oxidized low-density lipoprotein receptor (LOX-1), and the phosphorylation on Tyr^{1214} of VEGF receptor-2 (VEGFR-2).

A, The experimental procedure is described in the legend for Figure 5. On day 17, human umbilical vein endothelial cells (HUVECs) were fixed and submitted to immunostaining with anti-CD31 antibody for HUVECs to detect *in vitro* angiogenesis, which was assessed by the formation of capillary-like structures of HUVECs cocultured with PBMCs (top). On day 17, human aortic endothelial cells (HAECs) were submitted to immunoblotting for LOX-1 expression. HAECs were extracted in sample buffer containing β-mercaptoethanol. Ten μg of proteins were loaded on SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting. Note the marked increase in LOX-1 protein in HAECs cocultured with Ang II-stimulated PBMCs. Incubation of HAECs with valsartan (Val), but not soluble VEGFR-2 (sVEGFR-2), inhibits Ang II-stimulated PBMCs- induced expression of LOX-1 protein (middle).

On day 17, HAECs were also fixed and submitted to in-cell western blotting for VEGFR-2 and phosphorylated VEGFR-2 with a Li-Cor Odyssey system. Replacement of PBMCs in inserts and medium was described in the procedure of the tube formation system. The image represents a 24-well two color in-cell western blotting with the 800 (green) and 700 (red) channels detecting total VEGFR-2 (as normalization) and phosphorylated VEGFR-2, respectively (bottom).

Lane 1, Endothelial cells (ECs) were cocultured with PBMCs from OSAS; *Lane 2~5*, cocultured with Ang II-stimulated PBMCs from control in the presence of Ang II (*Lane 3,* pretreatment with IgG as a control; *Lane 4,* pretreatment with valsartan; *Lane 5,* pretreatment with sVEGFR-2*)*; *Lane 6*, ECs were cultured only in medium in the presence of VEGF as a positive control.

B, Ten different fields from the well were randomly selected, and tube formation was evaluated and quantified by the Chalkley counting method. Ang II-stimulated PBMCs from control increased tube formation of HUVECs. PBMCs-mediated increased tube formation was inhibited by Val and the sVEGFR-2 pretreatment. Data are expressed as the mean ± SEM. *, $P<0.05$ compared with OSAS, Val, and sVEGFR-2.

C, In-cell western blotting showed that the phosphorylated VEGFR-2 was low in HAECs cocultured with PBMCs from control (Figure 5), but was increased markedly in HAECs cocultured with Ang II-stimulated PBMCs from control compared to OSAS. This increase was suppressed by incubation with Val and sVEGFR-2. Phosphorylated VEGFR-2 was normalized by the total VEGFR-2 expression. The fold increase in phosphorylation induced by Ang II-stimulated PBMCs or VEGF was calculated by comparison with the phosphorylation in HAECs cocultured with PBMCs from OSAS. Data are expressed as the mean ± SEM. *$P<0.05$ compared with OSAS, Val, and sVEGFR-2.

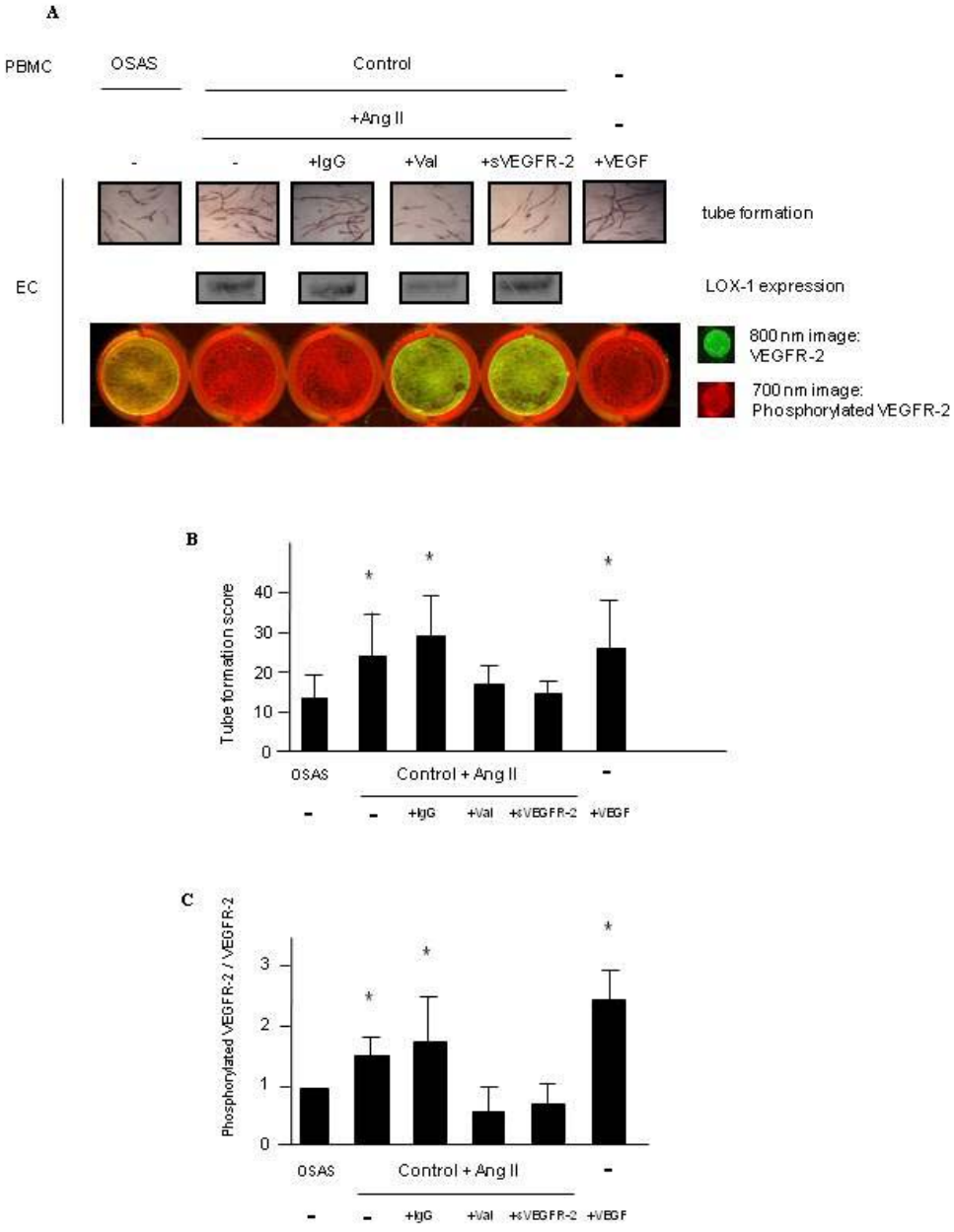
A
PBMC
OSAS
Control
+Ang II
-
-
-
-
+IgG
+Val
+sVEGFR-2
+VEGF
tube formation
EC
LOX-1 expression
800 nm image:
VEGFR-2
700 nm image:
Phosphorylated VEGFR-2
B
Tube formation score
0
10
20
30
40
OSAS
Control + Ang II
-
-
-
+IgG
+Val
+sVEGFR-2
+VEGF
C
Phosphorylated VEGFR-2 / VEGFR-2
0
1
2
3
OSAS
Control + Ang II
-
-
-
+IgG
+Val
+sVEGFR-2
+VEGF

Table 1. Demographic, sleep, and clinical data of control subjects and OSAS patients

	Control (n=38)	OSAS (n=37)	p*
Age, y	45.2 ± 2.3	46.4 ± 2.6	0.7
Body mass index, kg/m^2	30.0 ± 1.0	28.8 ± 1.0	0.4
Full blood count			
WBC, /μl	5926.3 ± 304.0	6167.6 ± 329.2	0.6
HB, g/dl	14.5 ± 0.2	14.9 ± 0.3	0.3
PLT, x10^4/ μl	26.0 ± 1.4	29.0 ± 1.3	0.1
Fasting lipids			
Total cholesterol, mg/dl	177.0 ± 5.0	185.0 ± 4.1	0.2
Triglyceride, mg/dl	105.0 ± 3.4	101.0 ± 3.6	0.4
HDL cholesterol , mg/dl	66.1 ± 1.8	64.8 ± 1.9	0.6
LDL cholesterol , mg/dl	89.8 ± 5.3	99.9 ± 4.1	0.1
Fasting Plasma glucose, mg/dl	98.2 ± 1.2	95.6 ± 1.4	0.2
Hemoglobin A1c, %	4.8 ± 0.1	4.9 ± 0.1	0.3
AST, IU/l	28.8 ± 1.4	27.1 ± 1.4	0.4
ALT, IU/l	23.7 ± 1.2	25.5 ± 1.6	0.4
Systolic Blood pressure, mmHg	123.7 ± 1.9	125.3 ± 2.0	0.6
Diastolic Blood pressure, mmHg	72.3 ± 1.6	74.3 ± 1.7	0.4
Sleep study details			
Apnea-hypopnea index	2.1 ± 0.2	37.2 ± 3.2	**<0.001**
Average nightly SaO_2,%	93.9 ± 0.5	83.3 ± 0.9	**<0.001**
Minimal SaO_2,%	88.3 ± 0.6	68.4 ± 1.3	**<0.001**
% total sleep time < 90%, %	2.0 ± 0.3	15.7 ± 1.1	**<0.001**

OSAS: obstructive sleep apnea syndrome; WBC : white blood cell; HB: hemoglobin; PLT: platelet; HDL: high-density lipoprotein; LDL: low-density lipoprotein; AST: aspartate transaminase; ALT: alanine aminotransferase; SaO_2: oxygen saturation

Values are expressed as mean ± SEM for each group.
*Bold values are statistically significant.

Table 2. Specific laboratory data for oxLDL and %CEPs

	Control	OSAS	post-CPAP
Plasma oxLDL (U/L)	32.3 ± 2.7	43.6 ± 3.6 *	32.7 ± 3.2
%CEPs (of white blood cell number)	0.48 ± 0.09	1.40 ± 0.33 *	0.75 ± 0.18

OSAS: obstructive sleep apnea syndrome; CPAP: continuous positive airway pressure; oxLDL: oxidized low-density lipoprotein; CEPs: circulating endothelial progenitor cells
Values are expressed as mean ± SEM for each group.
* p value<0.05 vs. Control and post-CPAP