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Novel Nox inhibitor of oxLDL-induced reactive oxygen species formation in human endothelial cells ☆

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Abstract

In this study, we investigated effects of a novel NAD(P)H oxidase (Nox)-inhibitor 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-*d*]pyrimidine (VAS2870) on oxidized low-density lipoprotein (oxLDL)-mediated reactive oxygen species (ROS) formation in human endothelial cells. Primary cultures of human umbilical vein endothelial cells were cultured to confluence and ROS formation was induced with 50 μg/ml oxLDL for 2 h. ROS formation was detected by chemiluminescence (CL) using the Diogenes reagent. OxLDL induced ROS formation in human endothelial cells ($171 \pm 12\%$; $n = 10$, $P < 0.05$ vs. control). This augmented ROS formation in response to oxLDL was completely inhibited by the Nox inhibitor VAS2870 ($101 \pm 9\%$; $n = 7$, $P < 0.05$ vs. oxLDL). Similar results were obtained with superoxide dismutase ($91 \pm 7\%$; $n = 7$, $P < 0.05$ vs. oxLDL). However, the Nox4 mRNA expression level was neither changed by oxLDL nor VAS2870. We conclude that VAS2870 could provide a novel strategy to inhibit the augmented endothelial superoxide anion formation in response to cardiovascular risk factors.

Keywords: VAS2870; oxLDL; NAD(P)H oxidase; Reactive oxygen species; Endothelial cells

The formation of reactive oxygen species (ROS) plays an important role in the pathology of vascular disorders [1]. Increased ROS formation in endothelial cells can reduce nitric oxide (NO) availability, interfere with redox-sensitive signalling cascades, and induce the oxidation of lipoproteins [2]. Therefore, increased vascular ROS formation can promote the development and progression of atherosclerosis [3]. Several molecular sources of endothelial ROS formation have been suggested [4,5]. Growing evidence supports NAD(P)H oxidase complexes as major sources of superoxide anions in endothelial cells [6–9].

The NAD(P)H oxidase complexes contain different catalytic Nox subunits [10]. Up to seven Nox isoforms have been described in different cell types [3,11]. The classical NAD(P)H oxidase complex is composed of a membrane-bound flavocytochrome b_{558} consisting of gp91^{phox}/Nox2 and p22^{phox} as well as cytosolic subunits (p47^{phox} and p67^{phox}) [6]. Besides the gp91^{phox}/Nox2-containing complex, Nox4 is the prominent Nox isoform in endothelial cell [7,9,12,13].

The proposed pathophysiological role of endothelial ROS formation urges the development of potent Nox inhibitors. All known chemical (e.g., diphenyliodonium chloride, DPI, or apocynin), peptide (e.g., gp91ds-tat), and genetic (e.g., siRNA) inhibition strategies of NADPH oxidase have limitations in specificity, stability, cellular

☆ *Abbreviations:* CL, chemiluminescence; HUVEC, human umbilical vein endothelial cells; Nox, NAD(P)H oxidase; oxLDL, oxidized low-density lipoprotein; SOD, superoxide dismutase; rLU, relative light units; ROS, reactive oxygen species.

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uptake, or in vivo availability. Therefore, development of novel non-peptide inhibitors is an important issue.

A novel Nox inhibitor suitable for the treatment of cardiovascular diseases should have the ability to reduce oxidative stress induced by pathophysiological stimuli, e.g., oxidized low-density lipoprotein (oxLDL), in human endothelial cells. In previous studies, we could demonstrate the induction of ROS formation in human endothelial cells after stimulation with oxLDL [9]. Therefore, we investigated the efficacy of the novel non-peptide Nox inhibitor VAS2870 [14] to reduce the oxLDL-induced ROS formation in primary cultures of human endothelial cells.

Materials and methods

Characterization of VAS2870 by NMR and mass spectrometry. The structure of the novel Nox inhibitor VAS2870 was characterized by NMR and mass spectrometry (^{1}H NMR (DMSO-d_6): δ 5.85 (s, 2H, CH_2), 7.3–7.4 (m, 5H, Ph), 7.5–7.6 (m, 2H, Ar), 7.85 (d, 1H, Ar), 7.95 (d, 1H, Ar), 8.95 (s, 1H, H-5). ms: (+APCl) m/z 361 $[M+H]^+$) (Fig. 1). VAS2870 has been identified as a inhibitor of NAD(P)H oxidases by high throughput screening. VAS2870 has an IC_{50} value of 2 μM in PMA-stimulated oxidative burst of HL-60 cells (not shown).

Cell culture. All cell culture reagents were purchased from Invitrogene (Karlsruhe, Germany). The chemicals were from Sigma–Aldrich (Munich, Germany) unless otherwise specified. Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated using collagenase II. To minimize variations of primary cultures, different batches of primary isolates were pooled and cultured on gelatine-coated plates in medium M199 supplemented with 10% fetal calf serum and 1% growth supplement. Before stimulation, confluent endothelial cells were incubated in serum-free medium for 5 h.

Stimulation of HUVEC with low-density lipoproteins. Native and oxidized low-density lipoproteins (nLDL, oxLDL) were isolated and characterized by standard procedures [15]. In brief, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were separated by centrifugation through NaBr gradients from human plasma. The LDL fraction was dialyzed against phosphate-buffered saline (PBS) and oxidized for 24 h using 50 μM $CuSO_4$. The oxidation level of LDL was analyzed by gel electrophoresis (Hydragel LDL/HDL CHOL Direct K20 kit, Sebia, Norcross, GA), measuring of increasing numbers of conjugated dienes in oxidized LDL by absorbance at 234 nm with a spectrophotometer (Biowave UV/Vis Diode Array Spectrophotometer, Biochrom, Cambridge, UK), and the Oxidized LDL ELISA (Mercodia, Uppsala, Sweden). Confluent primary cultures of HUVEC were stimulated with nLDL or oxLDL for the indicated times.

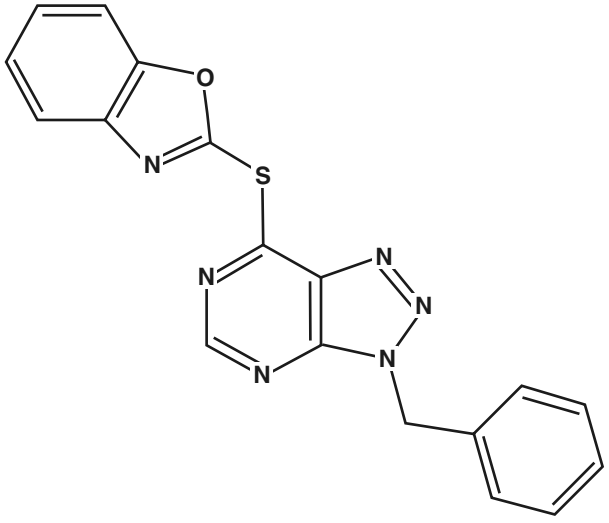

Fig. 1. Chemical structure of the Nox inhibitor VAS2870. The novel Nox inhibitor VAS2870 (3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-*d*]pyrimidine) was characterized by NMR and mass spectrometry.

Detection of reactive oxygen species. The sensitivity of different superoxide detection assays was compared using the xanthine/xanthine oxidase (X/XO) system. Superoxide generation was induced as previously described [16] with minor modifications. The superoxide anion generating reaction was initiated by addition of 500 μM xanthine to 0.1, 1, 10, or 100 mU/ml xanthine oxidase. Lucigenin (10 μM), coelenterazine (10 μM, Molecular Probes, Eugene, OR), Western Lighting (100 μl, Perkin Elmer, Boston, MA), or Diogenes™ Cellular Luminescence Enhancement System for Superoxide Detection reagent (100 μl, National Diagnostics, Atlanta, GA)-mediated chemiluminescence (CL) was measured in a FLUOstar OPTIMA multi-well reader (BMG, Offenburg, Germany). Addition of superoxide dismutase (SOD) from bovine erythrocytes (200 U/ml) stopped CL. The X/XO triggered CL was expressed as relative light units (rLU) (mean ± SEM of 4 independent experiments).

The formation of reactive oxygen species in response to lipoproteins was analyzed in HUVEC. After 2 h of stimulation without or with nLDL or oxLDL, the endothelial cells were detached using trypsin and transferred into Krebs–Henseleit solution (10 mM glucose, 0.02 mM Ca-Tritriplex, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 120 mM NaCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, and 5 mM KCl, pH 7.4). ROS formation was detected by chemiluminescence using the Diogenes reagent. The CL was measured over a period of 30 min in a FLUOstar OPTIMA multi-well reader (BMG, Offenburg, Germany). Results were given as CL in percent of control.

Inhibitor studies. Inhibition of oxLDL-mediated ROS formation in human endothelial cells was investigated using the Nox inhibitor 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-*d*]pyrimidine (VAS2870) (5 μM, vasopharm BIOTECH GmbH, Würzburg, Germany) [14], SOD (200 U/ml), or DPI (10 μM). After preincubation with or without the inhibitor for 0.5 h, oxLDL was added for 2 h. Each experiment was accompanied by internal controls from the same HUVEC preparation incubated for the same period of time without lipoproteins, but with inhibitors at identical concentrations. Controls without inhibitor were treated with the same amount of solvent.

RNA preparation and RT-PCR. After incubation with nLDL, oxLDL, or Nox inhibitor VAS2870 (5 μM), RNA was isolated using peqGOLD TriFast™ (peqlab Biotechnologie GmbH, Erlangen, Germany). In reverse transcriptase (RT) reactions, equal amounts of total RNA (500 ng) from HUVEC were incubated for 3 min at 70 °C, and subsequently reverse transcribed into cDNA using random hexamer primers and SuperScript™ III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) for 1 h at 42 °C. The Nox4 mRNA expression was determined by PCR using specific primers as previously described [17]. Expression was normalized to 18S rRNA.

Statistics. Data are given as means ± SEM, n indicates the number of independent experiments ($n \geqslant 3$). Statistical analysis was performed with the ANOVA procedure followed by Bonferroni's method (Sigma Stat 3.11, Systat Software, Richmond, CA). A value of $P < 0.05$ was considered statistically significant.

Results

Comparison of sensitivity of different superoxide detection systems

In order to improve the methods for determination of the potential of VAS2870, we first compared different CL superoxide detection systems using the X/XO system. Superoxide formation was measured using lucigenin,

coelenterazine, Western Lighting, or Diogenes reagent chemiluminescence. The specificity of superoxide anion formation and detection was confirmed by SOD. The sensitivity of superoxide detection increased in the order: lucigenin < coelenterazine < Western Lighting < Diogenes reagent (Fig. 2). The highest sensitivity reached the Diogenes reagent (approx. 200-fold higher than lucigenin) (Table 1). Therefore, this method was used in further studies with human endothelial cells.

OxLDL induces ROS formation in human endothelial cells

Human umbilical vein endothelial cells (HUVEC) were stimulated with nLDL or oxLDL (50 μg/ml) for 2 h. Subsequently, the formation of reactive oxygen species was measured by Diogenes chemiluminescence. Native LDL had no significant effect on ROS formation in HUVEC. In contrast, oxLDL significantly enhanced endothelial ROS formation to 171 ± 12% of time-matched internal control (Fig. 3).

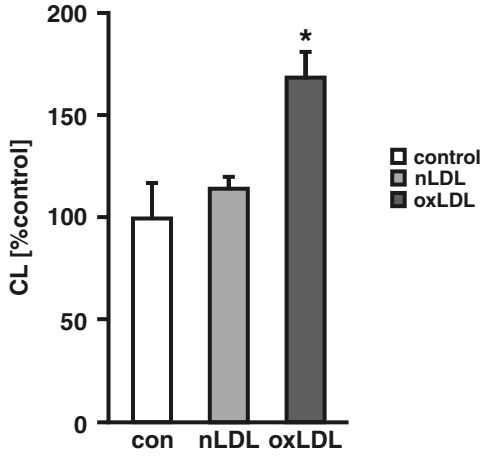

Fig. 3. Increased endothelial ROS formation in response to oxLDL. HUVEC were stimulated with nLDL or oxLDL (50 μg/ml) for 2 h and reactive oxygen species (ROS) formation was determined by Diogenes chemiluminescence (CL, in % of control). In contrast to native LDL (nLDL), oxidized LDL (oxLDL) increased ROS formation in human endothelial cells. $^{*}P < 0.05$ vs. control (con).

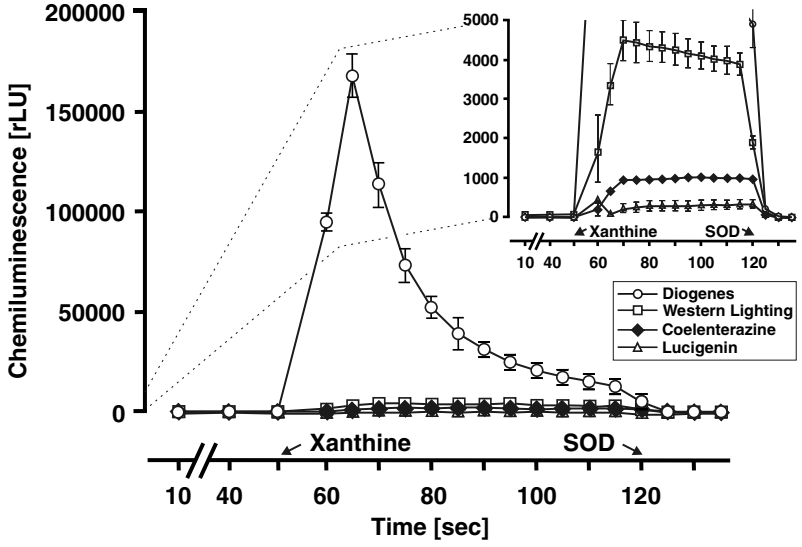

Fig. 2. Comparison of sensitivity of superoxide detection systems. The sensitivity of different superoxide detection systems was compared using the xanthine/xanthine oxidase system. The superoxide anion generating reaction was initiated by addition of xanthine (500 μM) to xanthine oxidase (10 mU/ml). Lucigenin (10 μM), coelenterazine (10 μM), Western Lighting (100 μl), or Diogenes (100 μl) reagent-mediated chemiluminescence was measured in relative light units (rLU) ($n = 4$). The specificity of the method was confirmed by superoxide dismutase (SOD).

Table 1
Sensitivity of detection of superoxide produced by the xanthine/xanthine oxidase system

Reagent	Xanthine oxidase (mU/ml)			
	100	10	1	0.1
Diogenes (100 μl)	217,403 ± 31,437	167,480 ± 10,849	12,493 ± 1519	255 ± 4
Western Lighting (100 μl)	37,519 ± 7472	4500 ± 514	715 ± 97	n.d.
Coelenterazine (10 μM)	6513 ± 135	1060 ± 96	53 ± 5	n.d.
Lucigenin (10 μM)	1082 ± 376	318 ± 96	n.d.	n.d.

Chemiluminescence of independent experiments ($n = 4$ each) are expressed as relative light units per minute. n.d., not detectable.

Inhibition of oxLDL-mediated ROS production by VAS2870

Initial experiments were performed to exclude morphological changes of the primary human endothelial cells due to the incubation with the novel Nox inhibitor VAS2870. The cell morphology after VAS2870 incubation for different periods of time was compared to the morphology of control cells. We did not observe any morphological changes of the endothelial cells with VAS2870 at concentrations $\leqslant 10$ μM (data not shown). Based on these findings the following experiments were performed with VAS2870 at concentrations of 5 μM.

The basal generation of ROS in unstimulated human endothelial cells was not affected by the novel Nox inhibitor VAS2870 (5 μM) ($95 \pm 10\%$ of control). However, the increased oxLDL (50 μg/ml)-mediated ROS production after 2 h was completely inhibited by preincubation with 5 μM VAS2870 ($101 \pm 9\%$, $P < 0.05$ vs. oxLDL) (Fig. 4). We further compared the inhibitory efficiency of VAS2870 with superoxide dismutase (SOD) and the flavoprotein inhibitor DPI. SOD showed no significant inhibition of ROS formation at basal level ($78 \pm 13\%$ of control), but it was able to inhibit the oxLDL-induced ROS formation ($91 \pm 7\%$, $P < 0.05$ vs. oxLDL) as well. In contrast to VAS2870, DPI reduced basal ROS formation in endothelial cells ($20 \pm 3\%$ of control, $P < 0.05$ vs. control). The oxLDL-stimulated endothelial ROS formation was inhibited by DPI as well ($32 \pm 7\%$ of control, $P < 0.05$ vs. oxLDL) (Fig. 4).

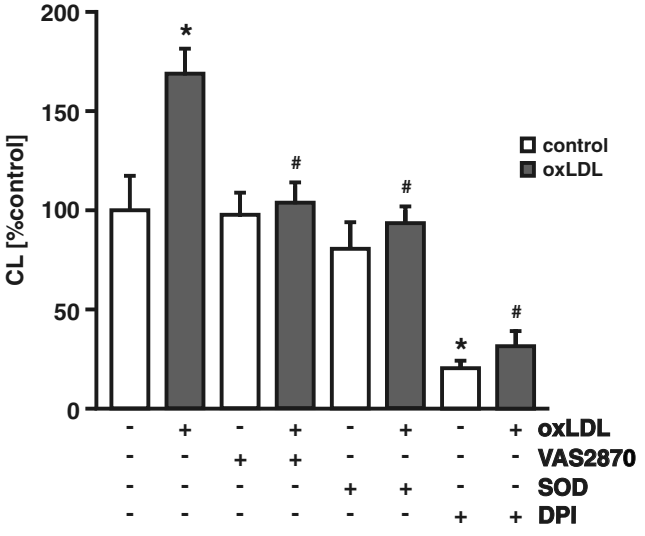

Fig. 4. Inhibition of oxLDL-induced ROS formation by the Nox inhibitor VAS2870 in human endothelial cells. HUVEC were incubated without lipoprotein (control), or stimulated with oxLDL (50 μg/ml) for 2 h and reactive oxygen species (ROS) formation was determined by Diogenes CL. The impact of VAS2870 (5 μM), SOD (200 U/ml), or DPI (10 μM) on endothelial ROS formation was determined in HUVEC without (control) or with oxLDL. VAS2870 reduced oxLDL-stimulated ROS formation in HUVEC. Values of chemiluminescence (CL) are given in percent of control. $^{*}P < 0.05$ vs. control (con), $^{\#}P < 0.05$ vs. oxLDL.

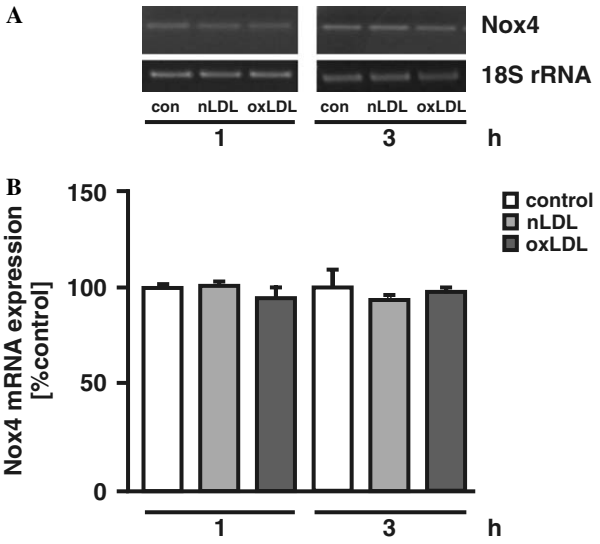

Fig. 5. Endothelial Nox4 mRNA expression in response to lipoproteins. Human endothelial cells (HUVEC) were incubated with native or oxidized LDL (nLDL, oxLDL, 100 μg/ml each) for 1 or 3 h. The mRNA expression of NAD(P)H oxidase subunit Nox4 was determined by quantitative reverse transcription-polymerase chain reaction and normalized to 18S rRNA RT-PCR (A). Values are given as means ± SEM in percentage of control (B). No significant changes in Nox4 mRNA expression were found.

Endothelial Nox mRNA expression in response to lipoproteins and VAS2870

In order to get further insight into the inhibitory mechanism of VAS2870, expression of the major endothelial Nox isoform Nox4 was measured in response to nLDL, oxLDL, and VAS2870. Native and oxidized LDL (100 μg/ml each) did not change the Nox4 mRNA expression in HUVEC after 1 and 3 h (Fig. 5). Furthermore, Nox4 mRNA expression level was not significantly changed by VAS2870 (5 μM) alone in human endothelial cells for up to 24 h (Fig. 6).

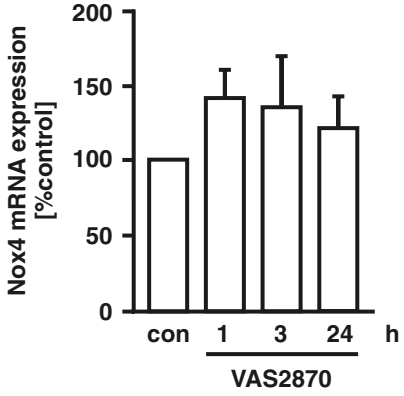

Fig. 6. Nox4 mRNA expression after application of VAS2870. Human endothelial cells (HUVEC) were incubated with VAS2870 (5 μM) for 1, 3, and 24 h. The mRNA expression of NAD(P)H oxidase subunit Nox4 was determined by quantitative reverse transcription-polymerase chain reaction and normalized to 18S rRNA RT-PCR. Values are given as means ± SEM in percentage of control. No significant changes in Nox4 mRNA expression were found compared to control.

Discussion

In this study, we show for the first time that the novel Nox inhibitor VAS2870 inhibits oxLDL-induced superoxide release from human endothelial cells. VAS2870 was not affecting basal endothelial superoxide formation. This Nox inhibitor has the advantage of a non-peptide inhibitor providing a better stability and in vivo administration compared to peptide inhibitors. The structure of VAS2870 represents a new class of Nox inhibitors [14].

In addition, we showed that the VAS2870 inhibitor had no impact on endothelial cell morphology at concentrations $\leqslant 10$ μM. Therefore, a non-specific inhibition due to effects on endothelial cell viability or function is unlikely.

In comparison to lucigenin, coelenterazine, and Western Lighting, the Diogenes reagent showed the highest sensitivity of superoxide detection and was therefore used for the measurement of ROS in human endothelial cells. In contrast to native LDL, oxidized LDL was able to induce an enhanced formation of superoxide anions in human umbilical vein endothelial cells. Enhanced vascular formation of reactive oxygen species in response to oxLDL has been described in several studies in vitro and in vivo [3,18]. This finding is in agreement with previous studies using the cytochrome c reduction assay [19]. The endothelial formation of superoxide anions by oxLDL is further supported by the inhibition with SOD and the flavoprotein inhibitor DPI. In contrast to DPI, VAS2870 did not reduce the ROS formation under basal conditions. This could be an advantage of VAS2870, because low levels of ROS are thought to play an important role in the physiology of vascular cells, e.g., by influencing signalling pathways [20].

The inhibitory effect of VAS2870 on oxLDL-induced endothelial ROS formation could be mediated by several putative sources of reactive oxygen species. Because NAD(P)H oxidases are considered as the major source of ROS in human endothelial cells, we focussed in this study on the expression of NAD(P)H oxidase subunits. Increasing evidence supports a preferential expression of Nox4 in endothelial cells [12,13]. Nox4 is also expressed in other vascular cell types and upregulated by angiotensin II in vitro and in transgenic models with an activated renin-angiotensin system in vivo [17]. Long-term exposure to oxLDL has been described to increase endothelial reactive oxygen species production and Nox4 mRNA expression in human coronary artery endothelial cells [21]. Because the ROS formation was already increased after 2 h, we focused on the short-term effects of oxLDL on Nox4. Nox4 mRNA expression was not increased after 1 or 3 h of incubation with oxLDL in HUVEC. Therefore, the increased ROS formation in response to oxLDL in this study most probably represents a functional activation of NAD(P)H oxidase complexes. In conclusion, a direct effect of VAS2870 on inhibition of NAD(P)H oxidase activity on the functional level could be postulated. This is further supported by our finding that VAS2870 has no effect on basal endothelial Nox4 expression.

In summary, we have shown for the first time that the novel NAD(P)H oxidase inhibitor 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-*d*]pyrimidine (VAS2870) efficiently inhibits oxLDL-mediated reactive oxygen species formation in human endothelial cells. This non-peptide inhibitor provides a novel strategy to inhibit the augmented endothelial superoxide anion formation in response to cardiovascular risk factors.

Acknowledgments

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