Pravastatin Inhibits Expression of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 (LOX-1) in Watanabe Heritable Hyperlipidemic Rabbits

A New Pleiotropic Effect of Statins

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Background—LOX-1, a receptor for oxidized low-density lipoprotein (OxLDL), seems to play a critical role in foam cell formation of macrophages (Mφs) and smooth muscle cells (SMC). Inhibition of LOX-1 expression reduces foam cell formation and might influence lipid core formation in atherosclerotic lesions. Because statins are able to downregulate LOX-1 expression in vitro, we examined if pravastatin can be used to reduce LOX-1 expression and lipid core formation in lesions of Watanabe heritable hyperlipidemic (WHHL) rabbits.

Methods and Results—Pravastatin downregulated LOX-1 expression in cultured human Mφs and in cultured human aortic SMCs. Homozygous WHHL rabbits were treated with 50 mg kg⁻¹ d⁻¹ pravastatin for 32 weeks. Immunohistochemical studies revealed that LOX-1 was expressed in intimal Mφs and SMCs of atherosclerotic lesions. The pravastatin-treated rabbits showed, compared with untreated rabbits, a significantly reduced LOX-1 protein and mRNA expression in the aortic arch. Lipid labeling of this aorta region also demonstrated a strong reduction of the ratio of lipid core area/total lesion area in pravastatin-treated rabbits.

Conclusions—The in vivo inhibition of LOX-1 expression by pravastatin demonstrated here represents a new pleiotropic effect of pravastatin. This in vivo inhibition of LOX-1 might be one mechanism for the lipid core reducing effect of pravastatin in atherogenesis. (Arterioscler Thromb Vasc Biol. 2006;26:604-610.)

Key Words: atherosclerosis ▪ cardiovascular disease prevention ▪ lipoproteins ▪ oxidized lipids ▪ smooth muscle cells

Oxidized low-density lipoprotein (OxLDL) is believed to play a key role in accumulation of foam cells in atherogenesis. The cellular uptake of OxLDL is mediated by so-called scavenger receptors, a heterogenous family of membrane bound proteins. Among them, the receptor LOX-1 is expressed in macrophages (Mφs) and smooth muscle cells (SMCs) in the intima of atherosclerotic lesions. Foam cells derive from Mφs and SMCs and have been implicated in the formation of the lipid core. Therefore, a role of LOX-1 in foam cell formation has been suggested, and downregulation of LOX-1 could in theory reduce the lipid core development by inhibition of foam cell formation.

Several authors have reported downregulation of LOX-1 expression in cultured cells in response to different stimuli. For example, upregulation of LOX-1 expression by angiotensin II can be inhibited by means of the angiotensin II type 1 receptor blocker losartan in endothelial cells (ECs). Upregulation of LOX-1 expression by tumor necrosis factor (TNF)-α and IL-1β is inhibited by peroxisome proliferator-activated receptor-γ (PPAR-γ) activators in SMCs and ECs. Furthermore, Chen et al (2000) showed that losartan decreased LOX-1 expression in cultured cells and also in the neointima of rabbits.

Lipid-lowering 3-hydroxy-3-methyl glutaryl coenzyme A (CoA) (3-hydroxy-3-methylglutaryl [HMG]-CoA) reductase inhibitors (statins) are also known to downregulate LOX-1. In cultured Mφ lovastatin inhibits LOX-1 expression in cultured Mφs. Li et al (2001) reported downregulation of LOX-1 expression and reduced uptake of OxLDL in EC after incubation with simvastatin and atorvastatin. It is known that statins are potent agents for lowering total and low-density lipoprotein cholesterol. Clinical trials have demonstrated that these agents are able to reduce the incidence of cardiovascular diseases. The risk of myocardial infarction of individuals treated with statins is significantly lower than that of individuals treated with other cholesterol-lowering agents. These
data suggest consequences of statin treatment beyond the well-known lipid-lowering effects. These so-called pleiotropic effects of statins include inhibition of SMC proliferation, anti-inflammatory effects, and downregulation of the scavenger receptors SR-A, CD36, and LOX-1.

Until now, no in vivo data on the influence of statins on LOX-1 expression in the vessel wall are available. Therefore, we examined the influence of pravastatin on LOX-1 expression and the progression of atherosclerotic lesions in Watanabe heritable hyperlipidemic (WHHL) rabbits. WHHL rabbits represent a well-accepted animal model for human atherosclerosis because these rabbits develop spontaneous atherosclerotic lesions with prominent lipid cores. Our study demonstrates that pravastatin downregulates LOX-1 expression in vivo. This new pleiotropic effect may contribute to the known beneficial influences of pravastatin treatment in atherogenesis.

Methods

Cell Culture and Reagents
Human aortic SMCs (third passage) were purchased from BioWhittaker and cultured in DMEM medium under a humidified atmosphere of 5% CO2/95% air at 37°C. Passages 4 to 6 were used for experiments. Pravastatin was kindly provided by Bristol-Myers Squibb, and mevalonate and geranylgeranyl pyrophosphate (GGPP) were from Sigma.

Reverse-Transcription Polymerase Chain Reaction
Total RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR) were performed as described previously. Briefly, total RNA from SMCs and aorta samples was isolated with RNasy Mini Kit (Qiagen) and reverse transcribed into cDNA (cDNA) using Superscript II (Invitrogen). PCR was performed with specific primers for human LOX-1 (forward: 5'-TTACTCTCATGTTGGTGC-3', reverse: 5'-AGCTTCTCTGCTTGTTGCC-3') and human 18S (forward: 5'-ACGACGACAGGTGACAAAGG-3', reverse: 5'-TTGGAGGTGCTGCTGAGGT-3'); human b-actin primers (forward: 5'-GGATCTCTCCTCAAGCTGAAAGG-3', reverse: 5'-GGGGTGTGAGGGTCTCAAA-3'); and human M-CSF (forward: 5'-GGATCTCTCCTCAGTTCAAG-3', reverse: 5'-GGGTGTGAGGGTCTCAAA-3') were used as internal standards. cDNA was amplified in a Stratagene thermocycler, and the number of cycles was individually selected for each pair of primers and each experiment. Measurements were performed to ensure that amplification took place within the linear range. Samples without reverse-transcriptase served as controls to exclude contamination with genomic DNA. The products were separated on ethidium bromide stained agarose gels and analyzed densitometrically.

Western Blot Analysis
The Western blot analysis was performed according to the method described in detail previously. Cells or aorta samples were lysed, ultrasonicated, and centrifuged at 5000 g. The samples were boiled and loaded onto a 10% SDS-polyacrylamide gel. Proteins were transferred by electroblotting, stained with Poncetin S to determine the amount of total protein, photographed, and blocked with ECL blocking agent (Amersham Biosciences). Membranes were incubated with polyclonal goat anti-LOX-1 antibody (Research Diagnostics), washed, and incubated with horseradish-conjugated anti-goat antibody (Vector Laboratories). Bound antibodies were detected by chemiluminescence using the ECL detection kit (Amersham Biosciences). The LOX-1 protein per lane was normalized to the total protein amount determined by Poncetin S staining.

Pravastatin Treatment of WHHL Rabbits
WHHL rabbits (3 months old, 14 males and 14 females) were obtained from the Centre de Production Animale (CPA). Animal housing and treatment was in accordance with the Guide for the Care

Hofnagel et al Pleiotropic Effect of Statins and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85-23, revised 1996); 14 animals (7 males and 7 females) were fed with pravastatin-enriched chow (50 mg kg⁻¹ d⁻¹ pravastatin in standard chow Ssniff K-H) and 14 animals (7 males and 7 females) were fed with unmodified standard chow (Ssniff K-H) for 32 weeks. Food intake was monitored every 3 days for both groups, and the body weights were determined every 4 weeks to exclude an influence of pravastatin treatment on eating behavior. Three animals of both groups died of otitis media at the age of 4 to 7 months.

Blood samples were taken from the marginal ear vein at the beginning of the experiment and after 16 and 32 weeks to measure total serum cholesterol levels. The levels were determined by using the enzymatic CHOL in vitro test kit (Roche).

All rabbits were killed after 32 weeks by administration of intravenous sodium pentobarbital (25 mg/kg), and aortas were prepared and subdivided into 5 regions (aortic arch, proximal thoracic aorta, distal thoracic aorta, proximal abdominal aorta, distal abdominal aorta). To perform immunohistochemical studies, serial cryosections (5- to 8-μm-thick) were used. For Western blot analysis and reverse-transcription PCR, samples were snap-frozen in liquid nitrogen (LN2) and homogenized using the ball mill Microdismembrator S (Braun Biotech) at 2.000 rpm for 1 minute.

Immunohistochemistry
Aortic cryosections from rabbits were fixed with ice-cold methanol, preincubated with 1% bovine serum albumin and incubated with monoclonal mouse anti-muscle actin antibody HHF35 (DAKO), monoclonal mouse anti-M6 antibody RAM11 (DAKO), or polyclonal goat anti-LOX-1 antibody (Research Diagnostics) at RT for 1 hour. Secondary anti-mouse or anti-goat antibodies conjugated with fluorochrome Cy3 (Dianova) were used. Sections incubated with mouse IgG (Dianova) served as controls. After nuclei staining with Hoechst dye 33258, the sections were analyzed with an Axioshot II fluorescence microscope (Zeiss).

Human aortic SMCs were fixed with 4% paraformaldehyde, preincubated with 1% bovine serum albumin, and incubated with polyclonal rabbit anti-nuclear factor (NF)-KB p65 antibodies (Santa Cruz), followed by Cy3-conjugated anti-rabbit secondary antibodies (Dianova). Cells incubated with rabbit IgG (Dianova) served for control.

Measurement of the Intima-Media Thickness and the Ratio of Lipid Core Area/Total Lesion Area
Aortic cryosections were used for the measurement of the intima-media thickness (IMT) and the lipid core area. The IMT was determined with the Zeiss Axioshot II fluorescence microscope and the morphometric software AnalySIS 3.0 (Soft Imaging Systems) using autofluorescence from collagen and elastin in the media. For quantification of the lipid core area, sections were stained with 1.8 mg/mL oil red O for 10 minutes, washed with H₂O and mounted with glycerine jelly. Analysis was performed with the Zeiss Axioshot II microscope and AnalySIS 3.0 software.

Results
Pravastatin Inhibits LOX-1 Expression in Cultured SMCs
Pravastatin downregulated LOX-1 expression in cultured human Møs (data not shown) and in cultured human aortic SMCs (Figure 1) in a dose- and time-dependent manner. Incubation with 50 μmol/L and 100 μmol/L pravastatin for 14 hours significantly decreased expression of LOX-1 mRNA (Figure 1a) and LOX-1 protein (Figure 1c). Incubation with 50 μmol/L pravastatin caused a transient decrease of LOX-1 mRNA with a maximum at 6 hours (Figure 1b). LOX-1 protein expression (Figure 1d) was decreased after incubation with 50 μmol/L pravastatin for 14 hours by 47%.
decrease of LOX-1 mRNA by pravastatin was completely prevented by addition of mevalonate or GGPP (Figure I, available online at http://atvb.ahajournals.org).

Localization of NF-κB Is Modulated by Pravastatin
The 5'-regulatory region of the LOX-1 gene contains a NF-κB-binding site. Therefore, we investigated if NF-κB could be involved in the pravastatin-mediated downregulation of LOX-1 expression we observed in cultured human aortic SMCs. We found significantly increased expression of inhibitory IκBα mRNA (Figure 2a) and reduced expression of NF-κB protein in the nucleus after incubation with 50 µmol/L pravastatin for 14 hours (Figure 2b).

Treatment of WHHL Rabbits With Pravastatin
WHHL rabbits were treated with pravastatin within the food (50 mg kg⁻¹ d⁻¹) for 32 weeks. Weights of the control animals (n=11) and of the pravastatin-treated rabbits (n=11) were comparable at the start of the experiment (2.8 kg±0.3 kg and 2.8 kg±0.4 kg). After 32 weeks, the weight of pravastatin-treated animals compared with that observed in control animals did not differ significantly (4.5 kg±0.5 kg and 4.2 kg±0.4 kg). Thus, pravastatin treatment did not influence the eating behavior of the rabbits.

Pravastatin Reduces Total Serum Cholesterol Levels of WHHL Rabbits
To ensure that in our experimental setting pravastatin exerted its well-known lipid-lowering effect, we determined total serum cholesterol levels. The levels were significantly decreased by pravastatin treatment: We found a reduction of 22% after 16 weeks and a reduction of 31% after 32 weeks in pravastatin-treated rabbits compared with the control group (Figure II).

Pravastatin Inhibits LOX-1 Expression in the Aorta of WHHL Rabbits
Pravastatin not only inhibited LOX-1 expression in cultured SMCs but also decreased expression of LOX-1 mRNA and LOX-1 protein in the aorta of pravastatin-treated WHHL rabbits (Figure 3). In the aortic arch region, LOX-1 mRNA expression was reduced by 21% (Figure 3a) and LOX-1 protein expression was reduced by 27% (Figure 3b). Furthermore, LOX-1 protein expression was decreased in the distal thoracic region of the aorta by 46% (Figure 3b). LOX-1 expression in the proximal thoracic and the abdominal regions of the aorta were not significantly influenced by pravastatin.

LOX-1 Protein Is Expressed in Intimal SMCs and Møs of WHHL Rabbit Aorta
We used immunohistochemistry to determine whether treatment with pravastatin modulates the distribution of LOX-1
protein in the vessel wall of WHHL rabbit aorta. Serial sections of the aortic arch from untreated and pravastatin-treated rabbits with advanced atherosclerotic lesions showed spindle-shaped SMCs in the media and in the fibrous cap of the lesion (Figure 4a, a'H11032) and round M'H9278s in the shoulder region of the lesion (Figure 4b, b'H11032). LOX-1 expression was detected in spindle-shaped cells corresponding to regions with SMC staining and in spherical cells corresponding to regions with M'H9021staining (Figure 4c, c'H11032). LOX-1 protein expression was not found in the media (Figure 4c, c'H11032). The distribution of LOX-1 protein in pravastatin-treated WHHL rabbits was compared with that found in untreated WHHL rabbits. In sections of rabbit aorta without atherosclerotic lesions or with early lesions, LOX-1 expression was observed mainly in endothelial cells. Staining of sections incubated with mouse IgG instead of specific antibodies was negligible.

**Pravastatin Reduces IMT and the Ratio of Lipid Core Area/Total Lesion Area in the Aorta of WHHL Rabbits**

Pravastatin treatment significantly reduced the IMT in all regions studied except for the distal thoracic region (Figure 5a). The influence of pravastatin was particularly evident in the aortic arch, showing 86% reduced IMT compared with the untreated control group. On average, the IMT of the whole aorta was decreased by 66% compared with the control group.

Furthermore, we evaluated the area of lipid cores (Figure 5b). In pravastatin-treated animals the ratio lipid core area/total lesion area was reduced. This reduction was significant in all regions of the aorta, except for the distal abdominal part. The maximum effect was found in the aortic arch, with a reduction of the ratio by 45%.

**Discussion**

**Pravastatin Inhibits LOX-1 Expression in Cultured M'H9278s and SMCs**

LOX-1 is assumed to play a key role in the uptake of OxLDL and subsequent foam cell formation of M'H9278s and SMCs in atherogenesis. Therefore, our study examined the potential of pravastatin to reduce LOX-1 expression and subsequent atherogenesis.

Draude et al8 described downregulation of LOX-1 expression in M'H9278s after incubation with lovastatin. Furthermore, it is known that in EC, LOX-1 expression can be inhibited by 2 other statins, atorvastatin and simvastatin.9,17,18 The present study shows for the first time to our knowledge that LOX-1 expression in SMCs is also downregulated by statins. This effect was prevented not only by addition of mevalonate, the primary product of HMG-CoA reductase, but also by GGPP, which is a downstream metabolite within the isoprenoid pathway. GGPP has been shown to be involved in the prenylation of proteins like RhoA, Cdc42, or Rac.11 Thus, we suggest that the pravastatin-induced changes in LOX-1 ex-
pression we observed are caused by impaired prenylation of these proteins.

Furthermore, our results indicate that NF-κB might be involved in the regulation of LOX-1 expression. The mammalian transcription factor NF-κB is known to regulate a wide variety of genes, including those involved in cell development and inflammation. The unactivated NF-κB/IKB complex is localized in the cytosol. After phosphorylation and degradation of IκB, NF-κB moves into the nucleus and stimulates the transcription of specific genes. Aoyama et al. investigated the structure of the LOX-1 gene and described a sequence reported to act as NF-κB binding site in the 5′ flanking region of the LOX-1 gene. We suppose that the expression of the LOX-1 gene is regulated by this mechanism. This is strengthened by the fact that we found a correlation between increased inhibitory IκB mRNA expression, reduced NF-κB protein expression in the nucleus and reduced LOX-1 expression after incubation of human aortic SMCs with pravastatin. The lipophilic atorvastatin and simvastatin seem to use the same mechanism as the hydrophilic pravastatin, because it is known that both lipophilic statins also decrease expression of NF-κB and LOX-1. The prenylation of proteins like Rho, Cdc42, or Rac might be a link between the inhibition of the isoprenoid pathway by statins and the inhibition of the NF-κB activity by statins, because it is known that these proteins are involved in NF-κB activation.

Figure 4. Immunohistochemistry of untreated (a to c) and pravastatin-treated (a’ to c’) WHHL rabbits. Cryosections of the aortic arch with advanced atherosclerotic lesions were used. Red staining represents the localization of spindle-shaped SMCs in the media and in the fibrous cap of the lesion (a and a’, arrowheads) and of round MΦs in the shoulder region of the lesion (b and b’, arrows). LOX-1 protein was found in spindle-shaped cells corresponding to SMC staining (c and c’, arrowheads) and in round cells corresponding to MΦ staining (c and c’, arrows). The LOX-1 protein distribution in pravastatin-treated WHHL rabbits appeared unchanged compared with untreated WHHL rabbits. Green staining is autofluorescence from collagen and elastin in the media (M), blue staining is caused by nuclei after incubation with Hoechst dye 33258. I indicates intima. Bar, 100 μm.

Figure 5. Aortic intima-media thickness (IMT) and ratio of lipid core area/total lesion area of untreated and pravastatin-treated WHHL rabbits. The medial and the intimal area of aortic cryosections were determined using fluorescence microscopy and morphometric analysis software. The morphometric determination of the lipid core area/total lesion area ratio was performed with oil red O-stained aortic cryosections. The IMT and the ratio of lipid core area / total lesion area were decreased in pravastatin-treated animals compared with untreated control animals. Values for each bar are means±SEM (n=11). n.s. indicates not significant. *P<0.05 vs control. **P<0.01 vs control.
The proinflammatory cytokines IL-1α, IL-1β, and TNF-α co-localize with LOX-1 expression in intimal SMCs of advanced atherosclerotic lesions and NF-κB is known to be strongly activated by these cytokines. Thus, we suppose that increased LOX-1 expression in atherogenesis caused by inflammatory stimuli as well as down-regulation of LOX-1 expression by statins is mediated by NF-κB.

**Pravastatin Inhibits LOX-1 Expression In Vivo**

Our studies clearly demonstrated the inhibition of LOX-1 expression by pravastatin in vitro, but it still remained unclear if pravastatin treatment exerts the same effects on LOX-1 expression and subsequent foam cell formation in vivo. The contribution of LOX-1 to foam cell formation of M₉₀s and SMCs is still undergoing discussion, but there is strong evidence that LOX-1 plays a critical role in atherogenesis: LOX-1 expression is highly upregulated in atherosclerotic lesions²⁻⁵ and activated M₉₀s.²² Moreover, in cultured M₉₀s and SMCs, blocking of LOX-1 activity by small interfering RNA (siRNA) or antibodies reduced uptake of OxLDL and foam cell formation.²³,²⁴

Because no previous studies have been conducted on the influence of pravastatin on LOX-1 expression in vivo, we performed in vivo experiments and demonstrated that pravastatin-treatment downregulated LOX-1 mRNA and protein expression in the aortic arch. Using New Zealand White rabbits, Chen et al.⁷ showed markedly increased IMT and strong LOX-1 expression after high-cholesterol diet. In contrast, control animals featured normal aortic structure with minimal LOX-1 expression. Unfortunately, Chen et al.⁷ did not determine the types of LOX-1 expressing cells. Our immunohistochemical experiments revealed internal intimal SMCs and M₉₀s to be the cell types with most prominent LOX-1 expression in advanced atherosclerotic lesions. In rabbit aortas without atherosclerotic lesions or with early lesions, LOX-1 expression was observed mainly in endothelial cells. We conclude that LOX-1 is involved in foam cell formation of SMCs and M₉₀s mainly in advanced lesions. LOX-1 expression in intimal M₉₀s and SMCs of advanced human atherosclerotic lesions was previously described by Kataoka et al.,² and these authors supported the hypothesis that LOX-1 may play a significant role in foam cell transformation of M₉₀s and SMCs. Our results showed LOX-1 expression in cultured human aortic SMCs and in intimal SMCs of atherosclerotic lesions, but we found no expression in medial SMCs. This might be caused by the SMC phenotype, because it is known that nonproliferative medial SMCs have a contractile phenotype, whereas proliferative cultured SMCs and intimal SMCs have a synthetic phenotype.²⁵ Therefore, we suggest that LOX-1 is expressed only in SMCs having a synthetic phenotype.

If the formation of foam cells in advanced lesions is inhibited by a reduction of LOX-1 expression, consequences for the lesion morphology would be expected. In particular, the formation of the lipid core in advanced lesions should be influenced, because necrotic foam cells most probably contribute to lipid core formation by releasing their lipid contents.²⁶⁻²⁸ In the aorta of pravastatin-treated rabbits, a reduction of the ratio lipid core area/total lesion area in regions with reduced LOX-1 expression was observed. The effects of pravastatin on formation of atherosclerotic lesions in WHHL rabbits have previously been described.²⁹⁻⁻³² Kroon et al.²⁹ treated WHHL rabbits with up to 40 mg kg⁻¹ d⁻¹ pravastatin and determined a 52% lower IMT in the whole aorta compared with untreated rabbits. Our experiment showed a reduction of the IMT by 66%, probably because of the higher pravastatin concentration (50 mg kg⁻¹ d⁻¹). Other studies examined the influence of pravastatin in deposition of extracellular lipids in the aorta of WHHL rabbits compared with untreated animals. Shiomi et al.³⁰ described a reduction of lipid deposition by 33% in the aortic arch of pravastatin-treated rabbits. Interestingly, sections of carotids obtained by endarterectomy from patients who received 40 mg × d⁻¹ pravastatin for 3 months also featured a 66% reduction of lipid-rich area.³³ Our observation of a significant reduction of the lipid core area in the aortic arch of pravastatin-treated rabbits is consistent with these data. The findings clearly demonstrate that pravastatin is able to inhibit deposition of lipids and is able to reduce the lipid core area in atherogenesis.

It is difficult to determine to what extent the pravastatin-triggered reduction of the lipid core area is caused by a downregulation of LOX-1 expression. The reduction of the lipid core area can also be caused by inhibition of other scavenger receptors, eg, scavenger receptor class A, which are known to be inhibited by pravastatin. An additional key question is to what extent the lowering of plasma lipoproteins by pravastatin influences the development of the lipid core. As shown by our study, the strongest decrease of lipid core area was observed in the aortic arch, that region featuring the strongest decrease of LOX-1 expression. Thus, our data permit the suggestion that the lipid core development is influenced by LOX-1.

Our results corroborate the importance of the pleiotropic effects beyond the well-known lipid-lowering influence of statins. The inhibition of foam cell formation via reduced LOX-1 expression could play a major role in stabilization of advanced atherosclerotic lesions, because enlargement of the lipid core results in lesion rupture and consequently in arterial thrombosis.

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**Online Figures**

**Figure I.** LOX-1 mRNA expression after incubation with pravastatin, mevalonate or GGPP. The downregulation of LOX-1 mRNA expression after incubation with 50 µM pravastatin for 14h was completely prevented by addition of mevalonate or GGPP. Values for each bar are means ± SEM (n=3). n.s.= not significant vs. control, *: P<0.05 vs control.

**Figure II.** Total serum cholesterol of untreated WHHL rabbits and pravastatin-treated WHHL rabbits. Three-month-old WHHL rabbits were or were not treated with 50 mg d^{-1} kg^{-1} pravastatin. Total serum cholesterol was determined at the beginning of the experiment and after 16 and 32 weeks. Total serum cholesterol was significantly reduced by pravastatin after 16 and 32 weeks. Values for each bar are means ± SEM (n= 11). n.s.= not significant, *: P<0.05 vs control.