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TNFα and oxLDL Reduce Protein *S*-Nitrosylation in Endothelial Cells*

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Nitric oxide (NO) plays an important role in the regulation of the functional integrity of the endothelium. The intracellular reaction of NO with reactive cysteine groups leads to the formation of *S*-nitrosothiols. To investigate the regulation of *S*-nitrosothiols in endothelial cells, we first analyzed the composition of the *S*-nitrosylated molecules in endothelial cells. Gel filtration revealed that more than 95% of the detected *S*-nitrosothiols had a molecular mass of more than 5000 Da. Moreover, inhibition of *de novo* synthesis of glutathione using *N*-butyl-sulfoximine did not diminish the overall cellular *S*-NO content suggesting that *S*-nitrosylated glutathione quantitatively plays only a minor role in endothelial cells. Having demonstrated that most of the *S*-nitrosothiols are proteins, we determined the regulation of the *S*-nitrosylation by pro-inflammatory and pro-atherogenic factors, such as TNFα and mildly oxidized low density lipoprotein (oxLDL). TNFα and oxLDL induced denitrosylation of various proteins as assessed by Saville-Griess assay, by immunostaining with an anti-*S*-nitrosocysteine antibody, and by a Western blot approach. Furthermore, the caspase-3 p17 subunit, which has previously been shown to be *S*-nitrosylated and thereby inhibited, was denitrosylated by TNFα treatment suggesting that *S*-nitrosylation and denitrosylation are important regulatory mechanisms in endothelial cells contributing to the integrity of the endothelial cell monolayer.

Nitric oxide (NO)[1] plays an important role in the regulation of the functional integrity of the endothelium, which acts as a barrier between the circulating blood and the underlying tissue (1, 2). In endothelial cells, nitric oxide is continuously synthesized by the endothelial NO synthase. NO is implicated in several physiological functions within the endothelium. Thus, NO regulates the vascular tone, provides anti-thrombotic and anti-inflammatory activity, and inhibits endothelial cell apoptosis (2–4).

NO can act via cGMP-dependent and independent pathways. During the last years, increasing evidence suggests that cGMP-independent processes are importantly contributing to the regulation of cellular signaling. Specifically, the *S*-nitrosylation of SH-groups by NO has been shown to contribute to cGMP-independent effects of NO (5–7). *S*-nitrosylation of caspases has been described to inhibit apoptosis signaling (8–10). Caspase-3 has been shown to be *S*-nitrosylated at cysteine 163, which resulted in an inhibition of caspase-3 activity *in vivo* and *in vitro* (8, 11–14). Furthermore, the small G-protein $p21^{ras}$ and the MAP-kinase JNK are regulated by *S*-nitrosylation (15, 16). Another example is the *S*-nitrosylation of transcription factors like NFκB, c-jun, and c-fos, which regulates gene expression underscoring *S*-nitrosylation as a widespread regulatory mechanism (for review see Ref. 17). Beside the *S*-nitrosylation of high molecular weight proteins, the formation of *S*-nitrosylated glutathione, GSNO, has been proposed to be one of the important storage forms for NO *in vivo* (18, 19).

Although various *S*-nitrosylated proteins and molecules have been identified, the regulation of the *S*-nitrosylation is not clear. A first hint for the potential reversibility of *S*-nitrosylation and thereby suggesting the process of denitrosylation as an important component of signal transduction cascades was reported by Mannick *et al.*, who demonstrated that *S*-nitrosylation of caspase-3 is reversible (20). Stimulation of human T and B cells with Fas, a known pro-apoptotic stimulus, led to denitrosylation of overexpressed caspase-3 (20). Meanwhile, further studies revealed that the glutathione-dependent formaldehyde dehydrogenase, which is conserved from bacteria to humans, specifically metabolizes GSNO to glutathione (21).

Therefore, we investigated the stability of GSNO and high molecular weight proteins in endothelial cells. The present study demonstrates that TNFα and mildly oxidized low density lipoprotein (oxLDL) reduce *S*-nitrosothiols in endothelial cells. The majority of *S*-nitrosothiols (95%) was associated with high molecular weight proteins suggesting a minor contribution of GSNO. TNFα and oxLDL induced denitrosylation of many proteins including the p17 subunit of caspase-3 suggesting that the mechanism of *S*-nitrosylation/denitrosylation plays an important regulatory role in endothelial cells.

MATERIALS AND METHODS

Cell Culture—Human umbilical vein endothelial cells were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for at least 18 h. Cells were incubated with 10 μg/ml oxLDL or 100 ng/ml TNFα as described previously (11, 22).

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[1] The abbreviations used are: NO, nitric oxide; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; GSNO, *S*-nitrosylated glutathione; TNFα, tumor necrosis factor α; oxLDL, mildly oxidized low density lipoprotein; wt, wild type; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-phenylidole; DAN, 2,3-diamino-naphthalene; MMTS, methyl-methanthiosulfate; biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; L-NMMA, N^G-monomethyl-L-arginine; L-NAME, N^G-nitro-L-arginine-methyl ester; BSO, butyl-*N*-sulfoximine; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase.

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Transfection—The plasmid encoding the caspase-3 p17 wt subunit and the caspase-3 p17 mutant type (C163S) were cloned as previously described (11). Human umbilical vein endothelial cells were transfected with 3 μg of plasmid and 25 μl of Superfect as described previously with a transfection efficiency of 40% (11).

S-NO Content—*S*-NO content was measured using the Saville-Griess assay as described (23). In brief, human umbilical vein endothelial cells were lysed in Griess lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM KCl, 1% Nonidet-P40, 1 mM phenylmethylsulfonyl fluoride, 1 mM bathocuproinedisulfonic acid, 1 mM diethylenetriaminepenta-acetic acid, 10 mM *N*-ethylmaleimide), and 80 μg of cell lysate was incubated with 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in the presence or absence of 3.75 mM *p*-chloromercuribenzosulfonic acid for 20 min, and *S*-NO content was measured photometrically at 540 nm. The amount was calculated using defined GSNO concentrations as a standard.

Immunostaining of S-Nitrosylated Proteins—Cells were incubated with or without TNFα for 18 h and fixed with 4% paraformaldehyde for 15 min at room temperature. After permeabilization and blocking (permeabilization solution: 3% bovine serum albumin fraction V, 0.3% Triton X-100, 5% horse serum in phosphate buffered saline (PBS)), cells were incubated with anti-nitrosocysteine antibody (1:50) over night at 4 °C. After incubation with a biotin-conjugated anti-rabbit antibody (1:500), cells were labeled with streptavidin-fluorescein and visualized by fluorescence microscopy (magnification 1:40). Nuclei were stained with 4′,6-diamidino-phenylidole (DAPI; 0.2 μg/ml in 10 mM Tris/HCl, pH 7, 10 mM EDTA, 100 mM NaCl) for 10 min, and the resulting figures were merged. For negative control fixed and permeabilized cells were preincubated with 0.8% $HgCl_2$ for 1 h at 37 °C.

Separation of Low Molecular Thiols and Protein S-NO—Cell lysates (500 μg of total protein) were passed through G-25 Sephadex columns preequilibrated with Griess lysis buffer. Protein *S*-NO was separated from low molecular mass *S*-NO by desalting with 150 mM NaCl. *S*-NO content was detected by using the Saville-Griess assay as described above. Data were calculated as percent of protein loaded on the column.

Intracellular GSH Content—After the indicated times, cells were washed with PBS. After addition of 1% sulfosalicyclic acid cells were immediately shock frozen at −80 °C for 2 min and the resulting lysate was centrifuged for 5 min 15,000 × *g* at 4 °C. The supernatant was incubated with Tris-EDTA buffer (20 mM Tris, pH 7.4, 1 mM EDTA) and dithiobis(2-nitrobenzoic acid) at room temperature for 10 min, and the optical density was determined with a photometer at 412 nm. The pellet was resuspended in 10 mM Tris, pH 8, 0.32 M sucrose, 5 mM EDTA, 1% Triton, 1 mM phenylmethylsulfonyl fluoride for 30 min on ice and then centrifuged for 10 min at 15,000 × *g* at 4 °C. The total protein content of the probe was determined.

Detection of S-Nitrosylated Caspase-3 p17 Subunit—Detection of *S*-nitrosylated caspase-3 p17 subunit was performed as described (16). In brief, cells were lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet-P-40, 0.5% deoxycholic acid, 0.1% SDS and subjected to immunoprecipitation. Immunoprecipitates were washed twice with lysis buffer and two times with PBS. The pellet was resuspended in 500 μl of PBS. After addition of 100 μM $HgCl_2$ and 100 μM 2,3-diamino-naphthalene (DAN), samples were incubated in the dark at room temperature for 30 min, and 1 M NaOH was added. The generated fluorescent triazole from the reaction of 2,3-diamino-naphthalene with the NO released from caspase-3 p17 subunit was measured using an excitation wavelength of 375 nm and an emission wavelength of 450 nm. As negative control the Myc antibody alone in lysis buffer was immunoprecipitated. The resulting background fluorescence intensity was subtracted from each experiment.

Detection of S-Nitrosylated Proteins by Western Blotting—Detection of *S*-nitrosylated proteins was performed as described with slight modifications (24). In brief, cells were lysed in HENS buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 0.01 mM neocuproine and 1% SDS) for 20 min and centrifuged at 20,000 *g* for 15 min. 800 μg of total protein was incubated with 20 mM methyl-methanthiosulfate (MMTS) for 20 min at 50 °C and vortexed every 2 min for 5 s. MMTS was removed by protein precipitation with acetone. After resuspending the pellet in HENS buffer, 0.4 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) and 1 mM sodium ascorbate were added and incubated for 1 h at room temperature in the dark. Gel electrophoresis and Western blotting were performed in the dark. Biotinylated proteins were detected by using a horseradish peroxidase-linked streptavidin according to the manufacturer's protocol.

Statistics—Statistical analysis was performed with analysis of variance or student's *t* test (SPSS-Software; Excel software).

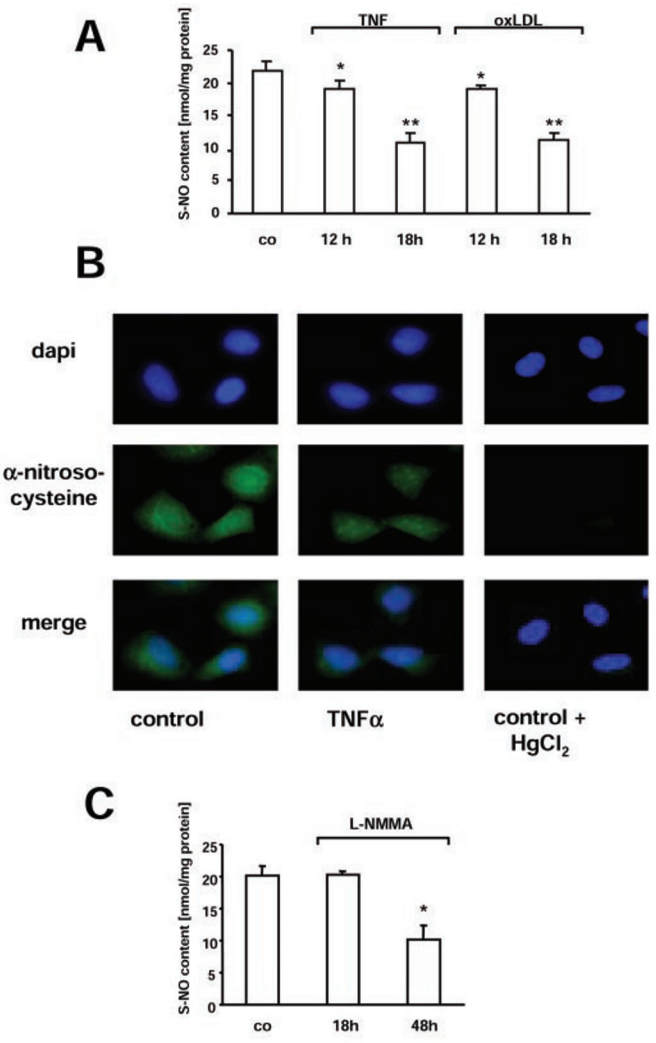

FIG. 1. *A,* TNFα and oxLDL reduced time dependently the *S*-NO content in endothelial cells. Endothelial cells were incubated for the indicated time points with TNFα (100 ng/ml) or oxLDL (10 μg/ml), and *S*-NO content was measured using the Saville-Griess Assay as described under "Materials and Methods." Data are means ± S.E., $n = 5$; $^*p < 0.05$ *versus* control (*co*); $^{**}p < 0.01$ *versus* control (*co*). *B,* immunostaining of *S*-nitrosylated proteins. Control cells and TNFα-treated cells (100 ng/ml, 18 h) were fixed and immunostained with anti-nitrosocysteine antibody as described under "Materials and Methods." *Upper panels* show staining of nuclei with DAPI, *middle panels* show staining for *S*-nitrosylated proteins visualized with streptavidin-fluorescein, and *lower panels* show the merged fluorescence signals. A representative figure is shown ($n = 3$). *C,* effect of NO synthesis inhibitor on *S*-nitrosylation in endothelial cells. Cells were incubated for the indicated times with L-NMMA (1 mM), and *S*-NO content was measured using the Saville-Griess Assay as described under "Materials and Methods." Data are mean ± S.E., $n = 4$; $^*p < 0.01$ *versus* control (*co*).

RESULTS

TNFα and oxLDL Reduce S-Nitrosothiol Content in Endothelial Cells—To address whether TNFα or oxLDL reduce the overall *S*-nitrosothiol content, endothelial cells were incubated with TNFα and oxLDL for 12 and 18 h. As shown in Fig. 1*A*, TNFα as well as oxLDL time dependently decreased *S*-nitrosothiols as measured by Saville-Griess assay. To confirm these findings, immunostainings were performed using an anti-*S*-nitrosocysteine antibody. Endothelial cells treated with TNFα and oxLDL showed significantly less *S*-nitrosocysteine compared with control cells. The specificity of the immunostaining was demonstrated by preincubation with $HgCl_2$ (Fig. 1*B* and data not shown). To exclude a possible involvement of TNFα-induced degradation of the eNOS mRNA and protein (25–27),

which might interfere with *de novo S*-nitrosylation, we incubated endothelial cells with N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of the eNOS, and measured the content of *S*-nitrosothiols. We could not detect any reduction of *S*-nitrosothiols after 18 h, but a significant decrease was determined after 48 h of incubation with L-NMMA (Fig. 1*C*) indicating a strong stability of the protein *S*-nitrosothiol adducts. Similar results were obtained using a different inhibitor of the eNOS, N^G-nitro-L-arginine-methyl ester (L-NAME), for 18 h (control: 20.3 ± 1.5 nmol *S*-NO/mg protein; L-NAME: 19.6 ± 1.6 nmol *S*-NO/mg protein). Moreover, coincubation of endothelial cells with TNFα and L-NMMA revealed similar results as for TNFα alone (9.9 ± 1.7 nmol *S*-NO/mg protein TNFα + L-NMMA *versus* 10.1 ± 1.4 nmol *S*-NO/mg protein TNFα, $n = 4$).

Characterization of Low and High Molecular Weight S-Nitrosothiols—To characterize the *S*-nitrosothiols involved in the *S*-nitrosylation/denitrosylation process in endothelial cells, we performed column chromatography using Sephadex G25 (cutoff: 5000 Da). As shown in Fig. 2*A*, the amount of low molecular weight *S*-nitrosothiols including GSNO was less than 5% of the overall *S*-NO content. These data indicate that proteins and not GSNO were denitrosylated upon stimulation with TNFα and oxLDL. To further underscore that GSNO is not the predominant form of NO in endothelial cells, cells were incubated with butyl-*N*-sulfoximine (BSO) to inhibit the *de novo* synthesis of GSH. BSO incubation did not reduce the overall *S*-NO content in endothelial cells (Fig. 2*B)*, although the GSH content was markedly decreased in the cells (Fig. 2*C*).

Regulation of Protein S-Nitrosylation/Denitrosylation—Having demonstrated that more than 95% of the *S*-nitrosothiols in endothelial cells are high molecular weight proteins, we characterized the pattern of *S*-nitrosylation using a Western blot approach. For this purpose, *S*-nitrosylated proteins were labeled with biotin using biotin-HPDP and detected with horseradish peroxidase-linked streptavidin. Incubation with TNFα showed a significant reduction of *S*-nitrosylated proteins (Fig. 3*A*). Quantification revealed that TNFα and oxLDL reduced *S*-nitrosylated proteins as shown in Fig. 3*B*. Equal loading of the blots was confirmed by reprobing the membranes with antibodies against the well established *S*-nitrosylated proteins, Ras and JNK (Fig. 3*A*). Moreover, bands with the proposed molecular mass for Ras (21 kDa) and JNK (46 kDa) seemed to be *S*-nitrosylated (Fig. 3*A*).

TNFα Induces Denitrosylation of Caspase-3 p17 Subunit in Endothelial Cells—To get further insights into specific targets, which are denitrosylated by TNFα, we investigated the caspase-3 p17 subunit. We and others showed previously that TNFα and oxLDL induced apoptosis in endothelial cells via activation of the cysteine protease family, the caspases, and, moreover, that *S*-nitrosylation of caspase-3 leads to its inactivation (8, 11–14, 28). Therefore, we hypothesized that TNFα induces denitrosylation of caspase-3 p17 subunit as an important mechanism for apoptosis induction in endothelial cells. To test this hypothesis, we transfected endothelial cells with Myc-tagged caspase-3 p17 wild type subunit (p17 wt) and the caspase 3 p17 mutant subunit, which contained a serine instead of a cysteine in its active center at position 163 (p17C163S). After stimulation with TNFα, the caspase-3 p17 subunit was immunoprecipitated, and *S*-nitrosylation was detected using the DAN reagent. Under control conditions, p17 wt was *S*-nitrosylated *in vivo*, which was significantly reduced in cells treated with TNFα (Fig. 3*C*). The data obtained for p17 wt after TNFα treatment were similar as for p17C163S without TNFα (Fig. 3*C*). These results demonstrate that TNFα induced denitrosylation of proteins, importantly the caspase-3 p17 subunit.

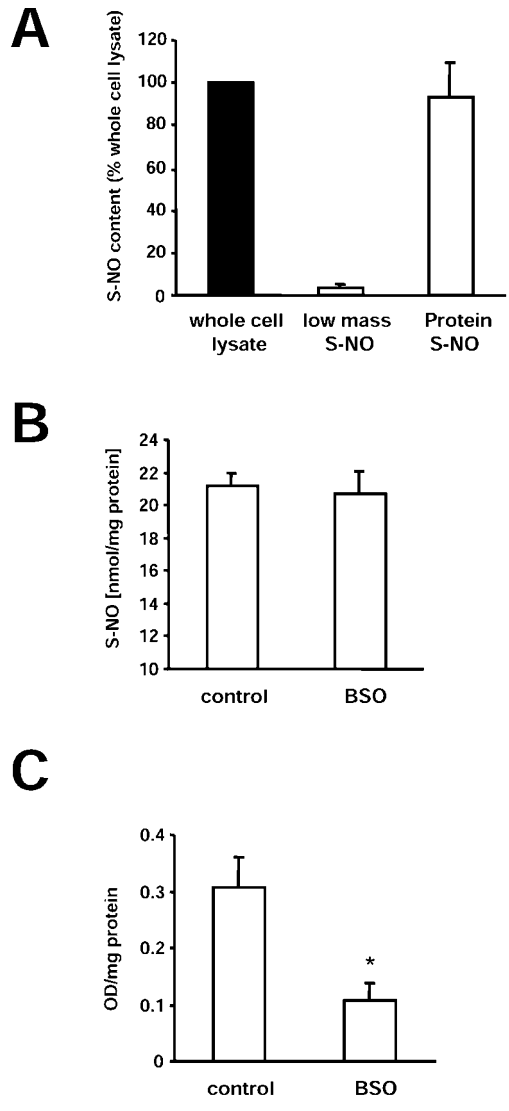

FIG. 2. *A*, characterization of *S*-nitrosothiols in endothelial cells. Low and high mass *S*-nitrosothiols were separated through a Sephadex G25 column by desalting. Data are mean ± S.E., $n = 3$. *B*, effect of BSO on *S*-NO content. Cells were treated with 10 μM BSO for 18 h, and *S*-NO content was measured using the Saville-Griess Assay as described under "Materials and Methods." Data are means ± S.E., $n = 5$. *C*, BSO reduced intracellular GSH content. Cells were treated with 10 μM BSO for 18 h, and GSH content was measured as described under "Materials and Methods." Data are mean ± S.E., $n = 5$; $^*p < 0.01$ *versus* control (co).

DISCUSSION

The present study demonstrates that TNFα and oxLDL can induce the denitrosylation of proteins in endothelial cells. The denitrosylation was demonstrated using different techniques including immunohistochemistry and the detection of *S*-NO with the Saville-Griess Assay. Furthermore, the recently developed method by Jaffrey *et al.* was used, which allows the detection of *S*-nitrosylated proteins by Western blot (24). Thereby, TNFα and oxLDL were shown to reduce the *S*-nitrosylation of various proteins. Furthermore, TNFα specifically denitrosylates the caspase-3 p17 subunit as demonstrated by the detection of the *S*-nitrosylation of p17 immunoprecipitates with the DAN assay. These data provide evidence for the denitrosylation as an important regulatory mechanism in endothelial cells.

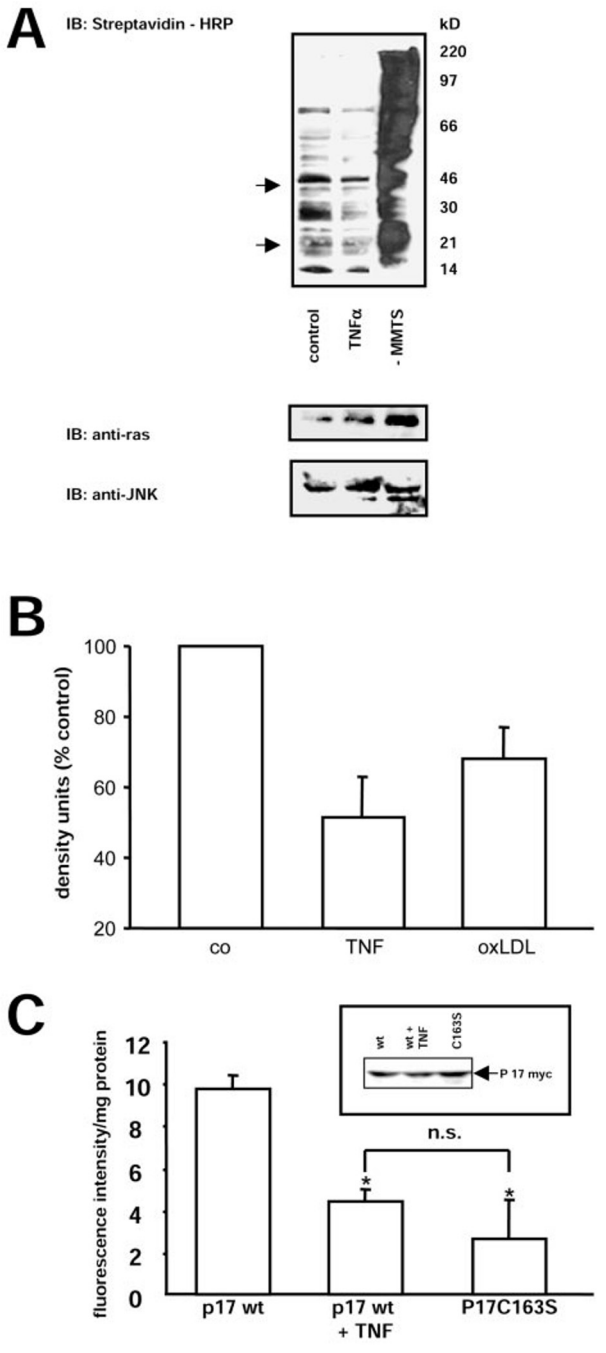

FIG. 3. *A,* TNFα reduced *S*-nitrosylated protein content in endothelial cells. After incubation with TNFα (100 ng/ml) for 18 h, *S*-nitrosylated proteins were determined by Western blot as described under "Materials and Methods." To detect all proteins containing an SH group independent of *S*-nitrosylation, cell lysates were incubated without MMTS as a positive control. To demonstrate equal loading, blots were stripped and reprobed with anti-JNK and anti-Ras antibodies. *Arrows* indicate possible *S*-nitrosylated bands for JNK and Ras. A representative Western blot is shown ($n = 4$). *B,* densitometric analysis of *S*-nitrosylated proteins. Extent of *S*-nitrosylation was quantified by scanning densitometry. Data are mean ± S.E., $n = 4$. *C,* denitrosylation of p17 wt by TNFα. Endothelial cells were transfected with Myc-tagged p17 wt or p17C163S. After treatment with TNFα for 18 h, *S*-nitrosylation was determined as described under "Materials and Methods." Data are mean ± S.E., $n = 4$; $^*p < 0.05$ *versus* p17 wt. Protein lysates were taken prior to immunoprecipitation to assure equal expression of p17 wt and p17C163S (*inset*).

The characterization of the *S*-nitrosothiols in endothelial cells revealed that most of the *S*-nitrosothiols were high molecular weight proteins as demonstrated by gel filtration. Moreover, inhibition of glutathione biosynthesis did not significantly affect the overall *S*-NO levels, suggesting that GSNO quantitatively does play a minor role. This is in accordance with a study by Eu *et al.*, who also demonstrated a minor contribution of GSNO to the overall *S*-NO levels in macrophages (23). A possible explanation of why GSNO did not accumulate in the cells could be a rapid metabolization. In support of this hypothesis, the thioredoxin system was shown to rapidly cleave NO out of GSNO in a cell-free system (29). In addition, a glutathione-dependent formaldehyde dehydrogenase was identified in eukaryotes, which specifically denitrosylates GSNO (21).

We and others showed previously that apoptosis induction in endothelial cells was dependent on the caspase cascade, and that predominantly caspase-3 activity was inhibited by direct *S*-nitrosylation of the cysteine 163 in the active center of the caspase-3 p17 subunit (8, 11–14, 28). First evidence that this process is reversible came from Mannick *et al.*, who reported a denitrosylation of the caspase-3 p17 subunit in iNOS-transfected B and T cells upon Fas-receptor stimulation (20). In line with these findings, the data of the present study revealed that the stimulation of endothelial cells with pro-inflammatory or pro-atherogenic stimuli reduced the *S*-nitrosylation of distinct proteins in endothelial cells and specifically reduced the *S*-nitrosylation of the p17 subunit of caspase-3. The decline of *S*-nitrosylation could be due to an inhibition of eNOS expression and/or NO bioavailability induced by TNFα-mediated eNOS mRNA destabilization (27) or increased oxidative stress (30, 31). However, pharmacological inhibition of NO synthesis for 18 h with L-arginine-L-NAME or L-NMMA did not decrease the *S*-NO levels, although control experiments confirmed the inhibition of eNOS (data not shown). These data indicate that the intracellular *S*-nitrosylation under basal conditions is very stable, which is in accordance with a recent study by Mannick *et al.* (20). Furthermore, the reduction of *S*-nitrosylation by incubation with TNFα or oxLDL seems to be caused by an active denitrosylation process rather than a decline in *de novo* *S*-nitrosylation. The molecular mechanism underlying this denitrosylation requires further investigation. In bacteria, constitutive and inducible enzymes exist, which cleave *S*-NO to NO (32, 33). Furthermore, a metabolic enzyme with GSNO reductase activity was identified in cytokine-stimulated macrophages, which also can reduce protein-*S*-NO (21). However, since GSNO is the preferred substrate (21), it is not clear whether this reductase can mediate TNFα- or oxLDL-induced denitrosylation.

The regulation of *S*-nitrosylation may importantly contribute to cellular signaling and endothelial function. Specifically, the inhibition of endothelial cell apoptosis has at least in part been attributed to the blockade of caspase via *S*-nitrosylation. The reversal of the *S*-nitrosylation may allow for caspase-activation, which is required for apoptosis signaling. Furthermore, *S*-nitrosylation of various other proteins was detected under basal conditions. The identity of these proteins needs to be defined in future studies. Given that small GTP-binding proteins, kinases, proteases and transcription factors are known to be *S*-nitrosylated, one may speculate that the reversal of the *S*-nitrosylation by pro-inflammatory or pro-atherosclerotic factors may play a crucial role in cellular signaling.

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