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-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.

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The abbreviations used are: NO, nitric oxide; INK, c-Jun NH₂-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-methanethiosulfate; biotin-HIPO; N⁶-[biotinamido(hexyl)-3'-2'-pyrididyldithio]propionamide; l-NMMA, N⁶-mono-methyl-l-arginine; l-NAME, N⁶-nitro-l-arginine-methyl ester; BSO, butyl-N-sulfoximine; ENOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase.

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Nitric oxide (NO) plays an important role in the regulation of the functional integrity of the endothelium. 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).
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 phenylmethylsulfon 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-chloromercuribenzenesulfonic 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) overnight 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-diamino-phenylindole (DAPI, 0.2 μg/ml in 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10 mM NaCl) for 10 min, and optical sections were determined by fluorescence microscopy. A representative figure was shown (n = 3), C, effect of NO synthesis inhibitor on S-nitrosothiol content, endothelial cells were incubated for the indicated times with 1-NMMA (100 ng/ml), and S-NO content was measured using the Saville-Griess Assay as described under “Materials and Methods.” Data are means ± S.E., n = 4; *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. Endothelial cells treated 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, **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. 1A, 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 HgCl2 (Fig. 1B and data not shown). To exclude a possible involvement of TNFα-induced degradation of the eNOS mRNA and protein (25–27),

FIG. 1. A, TNFα and oxLDL reduced time dependently the S-NO content in endothelial cells. Endothelial cells were incubated for the indicated times 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 = 4; *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. Endothelial cells treated 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, **p < 0.01 versus control (co). C, effect of NO synthesis inhibitor on S-nitrosothiol content in endothelial cells. Cells were incubated for the indicated times with 1-NMMA (10 μM), and S-NO content was measured using the Saville-Griess Assay as described under “Materials and Methods.” Data are means ± S.E., n = 4; *p < 0.01 versus control (co).
which might interfere with de novo S-nitrosylation, we incubated endothelial cells with N\textsuperscript{G}-monomethyl-l-arginine (l-NMMA), a competitive inhibitor of the eNOS, and measured the content of S-nitrosothiois. We could not detect any reduction of S-nitrosothiois after 18 h, but a significant decrease was determined after 48 h of incubation with l-NMMA (Fig. 1C) indicating a strong stability of the protein S-nitrosylation adducts. Similar results were obtained using a different inhibitor of the eNOS, \textit{A}\textsuperscript{3}-nitro-l-arginine-methyl ester (l-NNAME), for 18 h (control: 20.3 \pm 1.5 nmol S-NO/mg protein; l-NNAME: 19.6 \pm 1.6 nmol S-NO/mg protein). Moreover, coincubation of endothelial cells with TNF\textalpha and l-NMMA revealed similar results as for TNF\textalpha alone (9.9 \pm 1.7 nmol S-NO/mg protein TNF\textalpha + l-NMMA versus 10.1 \pm 1.4 nmol S-NO/mg protein TNF\textalpha, n = 4).

**Characterization of Low and High Molecular Weight S-Nitrosothiois**—To characterize the S-nitrosothiois involved in the S-nitrosylation/denitrosylation process in endothelial cells, we performed column chromatography using Sephadex G25 (cut-off: 5000 Da). As shown in Fig. 2A, the amount of low molecular weight S-nitrosothiois 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 TNF\textalpha 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 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 TNF\textalpha showed a significant reduction of S-nitrosylated proteins (Fig. 3A). Quantification revealed that TNF\textalpha and oxLDL reduced S-nitrosylated proteins as shown in Fig. 3B. Equal loading of the blots was confirmed by reprobing the membranes with antibodies against the well established S-nitrosylated proteins, Ras and JNK (Fig. 3A). Moreover, bands with the proposed molecular mass for Ras (21 kDa) and JNK (46 kDa) seemed to be S-nitrosylated (Fig. 3A).

**TNF\textalpha Induces Denitrosylation of Caspase-3 p17 Subunit in Endothelial Cells**—To get further insights into specific targets, which are denitrosylated by TNF\textalpha, we investigated the caspase-3 p17 subunit. We and others showed previously that TNF\textalpha 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 TNF\textalpha 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 TNF\textalpha, the caspase-3 p17 subunit was immunoprecipitated, and S-nitrosylation was detected using the DAN reagent. Under control conditions, p17 wt was S-nitrosylated \textit{in vitro}, which was significantly reduced in cells treated with TNF\textalpha (Fig. 3C). The data obtained for p17 wt after TNF\textalpha treatment were similar as for p17C163S without TNF\textalpha (Fig. 3C). These results demonstrate that TNF\textalpha induced denitrosylation of proteins, importantly the caspase-3 p17 subunit.

**DISCUSSION**

The present study demonstrates that TNF\textalpha 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 \textit{et al.} was used, which allows the detection of S-nitrosylated proteins by Western blot (24). Thereby, TNF\textalpha and oxLDL were shown to reduce the S-nitrosylation of various proteins. Furthermore, TNF\textalpha 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.
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 macophages (23). A possible explanation of why GSNO did not accumulate in the cells could be a rapid metabolism. In support of this hypothesis, the thioloredoxin 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, 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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