

# Erythropoietin Increases Asymmetric Dimethylarginine in Endothelial Cells: Role of Dimethylarginine Dimethylaminohydrolase

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Recombinant human erythropoietin therapy frequently causes hypertension in humans and animals with chronic renal failure. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthase, and its accumulation has been associated with reducing NO bioavailability and increasing superoxide generation. Whether epoetin  $\beta$  (EPO) or darbepoetin  $\alpha$  (NESP) can modify the levels of ADMA in endothelial cells was investigated. Endothelial cells from the third passage were incubated for 24 h in the presence of various concentrations of EPO or NESP (0, 0.1, 1, 10, 50, 100, and 200 U/ml). The levels of ADMA, allantoin, nitrate, and nitrite in conditioned media and the activity of dimethylarginine dimethylaminohydrolase (DDAH), the content of thiols and reactive oxygen species in endothelial cells, were determined. When endothelial cells were exposed to EPO or NESP, ADMA concentration in the cell culture medium increased significantly in a dose-dependent manner *versus* control. This effect was associated with a reduced activity of DDAH, the enzyme that degrades ADMA. Furthermore, EPO- or NESP-induced accumulation of ADMA was accompanied by a significant reduction of NO synthesis and an increase in oxidative stress. Both allantoin, a marker of oxygen free radical generation, and reactive oxygen species increased significantly after EPO or NESP treatment compared with control. The antioxidant pyrrolidine dithiocarbamate preserved DDAH activity and reduced ADMA accumulation in the same way as the co-incubation with anti-EPO neutralizing antibody. EPO and NESP posttranslationally impair DDAH activity via increased oxidative stress, causing ADMA as an important cardiovascular risk factor to accumulate and inhibit NO synthesis.

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Recombinant human erythropoietin (EPO) has been used in the treatment of anemia in chronic kidney disease since 1986 and is now used in the treatment of anemia of different causes (1). Darbepoetin  $\alpha$  (NESP) is a hyperglycosylated analogue of recombinant human EPO that has five additional amino acids in its primary sequence and two extra N-linked carbohydrate side chains, giving it a longer plasma half-life (2).

Although EPO exerts many beneficial effects in reversion of anemia, pharmacologic doses of EPO may show side effects arising from the vascular system (3). A frequently seen adverse effect of chronic administration of EPO is an increase in arterial BP in patients and experimental animals with chronic renal failure (4). Postulated mechanisms for EPO-induced hypertension include increased blood viscosity as well as enhanced vascular reactivity and vasoconstrictor responses. We demon-

strated previously that incubation with EPO increases the release of vasoconstrictive endothelin 1, prostaglandin  $F_{2\alpha}$  and thromboxane  $B_2$  and decreases the release of the vasodilator prostacyclin in isolated rabbit arterial rings and in endothelial cells (5). Nitric oxide (NO) can be another possible factor that contributes to hypertension after EPO treatment. Vaziri *et al.* (6) showed that EPO-induced hypertension may be causally related to impaired vasodilatory response to NO in rats. This finding is in agreement with studies of cultured human endothelial cells in which incubation with EPO decreased basal NO production and depressed NO synthetase expression (7).

Asymmetric dimethylarginine (ADMA), a novel cardiovascular risk factor (8,9), is an endogenous inhibitor of nitric oxide synthase (NOS). Elevations in plasma ADMA may contribute to the vascular pathophysiology observed in atherosclerosis, hypertension, hypercholesterolemia, and renal failure (10). Increased oxidative stress seems to play an important role in the pathogenesis of these clinical conditions. ADMA is actively metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (11). This enzyme seems to be exquisitely sensitive to oxidative stress, an effect that is likely responsible for ADMA accumulation. Collectively, the ADMA-DDAH system may be involved in the regulation of endogenous NO

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synthesis. Pharmacologic inhibition of DDAH leads to increased ADMA concentrations and a reduction in NO-mediated vasodilation, indicating that DDAH activity controls endogenous ADMA concentrations and thus NOS activity (12).

In this study, we hypothesized that EPO and NESP dysregulate the ADMA-DDAH system by increasing oxidative stress and thereby inhibit NO synthesis. To test this hypothesis, we examined the effects of antioxidant pyrrolidine dithiocarbamate (PDTC) on DDAH activity in EPO- or NESP-stimulated endothelial cells.

## Materials and Methods

### Cell Culture

Human umbilical vein endothelial cells (HUVEC; Cambrex, Kerviers, Belgium) were cultured in endothelial basal medium (Cambrex) supplemented with hydrocortisone (0.5 mg/ml), gentamicin (30  $\mu$ g/ml), amphotericin B (15  $\mu$ g/ml), human EGF (10  $\mu$ g/ml), human fibroblast growth factor-B (1  $\mu$ g/ml), vascular endothelial growth factor (2  $\mu$ g/ml), ascorbic acid (75 mg/ml), R<sup>3</sup>-IGF-1 (5  $\mu$ g/ml), heparin (1 mg/ml), and 2% FCS at a density of 6000 cells/cm<sup>2</sup> per 25- or 75-cm<sup>2</sup> flasks. The medium was replaced every second day. After detachment with trypsin/EDTA (Promocell, Heidelberg, Germany), endothelial cells were counted in a Neubauer cell chamber (Assistent, Sondheim, Germany) and the vitality was determined by means of staining with trypan blue (0.5%; Sigma, Tautkirchen, Germany) in physiologic saline. Viability after trypsinization was usually >95%. HUVEC were stained with von Willebrand factor to exclude dedifferentiation.

### Study Protocols

The cells that were obtained on third passage were used for the experiments. After reaching confluence, endothelial cells were treated with epoetin  $\beta$  (EPO; 0.1, 1, 10, 50, 100, and 200 U/ml; Hoffmann-La Roche, Mannheim, Germany), EPO plus 10  $\mu$ g/ml anti-EPO neutralizing antibody (EPO Ab; R&D Systems, Wiesbaden, Germany), EPO plus 10  $\mu$ M PDTC (Sigma), or vehicle for 24 h as indicated for each experiment. The studies were repeated with cells that were treated with NESP (0.1, 1, 10, 50, 100, and 200 U/ml; Amgen, Munich, Germany). At the conclusion of the 24-h treatment period, the cells and the supernatants were harvested and saved for the following measurements. The total cellular protein was measured using BCA protein assay kit (Pierce, Bonn, Germany).

### Determination of ADMA

For the determination of ADMA in cell culture supernatants, we adopted the HPLC-mass spectrometry method for plasma and urine published previously by our group (13). The calibration covers the range of 0.15 to 6  $\mu$ M. The calibration function was linear, and the limit of detection was found to be 0.05  $\mu$ M for ADMA. The intraday precision was 3.1%, and the interday precision was 3.3%.

### Measurement of Nitrate and Nitrite

The determination of nitrate and nitrite in cell culture supernatants was carried out in accordance to the method described by Tsikas *et al.* (14). In our laboratory, the intraday precision test yields a relative SD of 3.8% for nitrite and 1.3% for nitrate. The interday precision test yields a relative SD of 4.4% for nitrite and 4.2% for nitrate.

### DDAH Activity Assay

DDAH activity in endothelial cells was estimated by directly measuring the amount of ADMA metabolized by the enzyme described by

Lin *et al.* (15). The ADMA level in each group was measured by HPLC-MS as described above.

### Detection of Oxidative Stress

For the determination of allantoin, a marker of oxygen free radical generation, in cell culture supernatants, we adopted the method for human plasma described by Doehner *et al.* (16). In brief, after addition of the internal standard <sup>15</sup>N-allantoin, the mixture was evaporated in vacuum. The residue was derivatized with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide and analyzed by gas chromatography-mass spectrometry. The intraday precision test yields a relative SD of 2.1%, and the interday precision test yields a relative SD of 3.8%, with an accuracy of 2.8%.

The intracellular thiol concentration was measured by 5-chloromethylfluoresceindiacetate (CMFDA) staining in flow cytometry, as described previously (17). Briefly, cell samples were stained with CMFDA (Molecular Probes, Eugene, OR) at a final concentration of 12.5  $\mu$ M in PBS for 15 min at room temperature. After washing, the cells were fixed in 1% paraformaldehyde and analyzed within 2 h by flow cytometry at  $\lambda_{EX} = 490$  nm/ $\lambda_{EM} = 520$  nm (Epics XL-MCL; Coulter, Krefeld, Germany).

Dihydrorhodamin 123 (DHR) was used as a marker for intracellular reactive oxygen species (ROS). The cells were incubated for 20 min at 37°C in the presence of 10  $\mu$ M DHR with gentle agitation. The reaction was stopped by cooling on blue ice for 1 min and subsequent addition of 500  $\mu$ l of PBS followed by two washing steps. After a final fixation with 1% paraformaldehyde, the cells were analyzed by flow cytometry (Epics XL-MCL).

HUVEC were defined by forward/side scattering and live-gated for analysis. The levels of intracellular thiols and ROS were indicated by mean fluorescence intensities of stained probes *versus* negative controls. Fluorogenic beads (Flow check; Coulter), added to each sample, were used for internal calibration of the quantitative measurement.

### Statistical Analyses

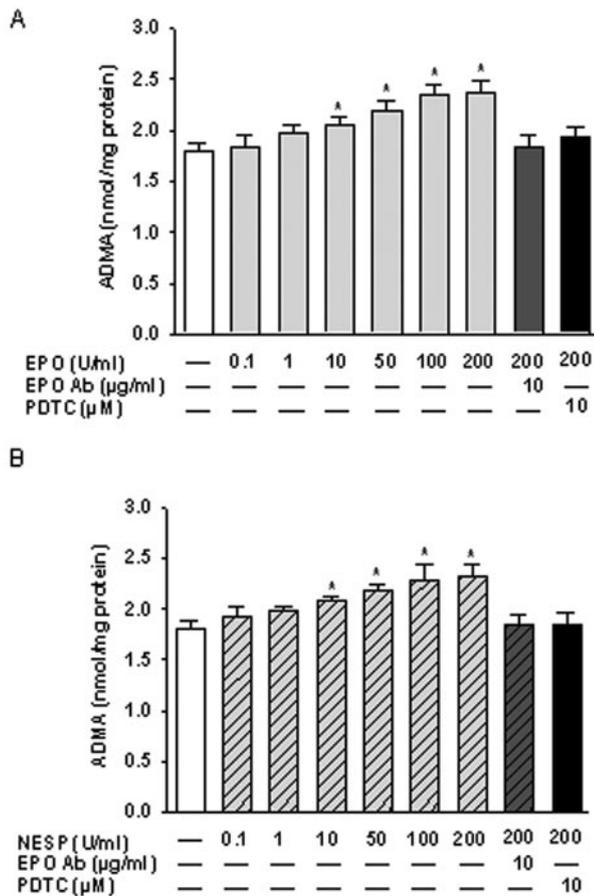
All data are given as mean  $\pm$  SEM from at least six independent experiments performed in duplicate. Statistical significance was tested with ANOVA using a LSD *post hoc* test or multiple comparisons (SPSS Software 11.0). Differences were considered significant at  $P < 0.05$ .

## Results

### Erythropoietin-Inhibited NO Synthesis Was Associated with an Accumulation of ADMA

Incubation of endothelial cells with EPO (0.1, 1, 10, 50, 100, and 200 U/ml) for 24 h resulted in a dose-dependent increase of ADMA in conditioned medium (Figure 1A). EPO at 1 U/ml had an initial but nonsignificant effect on ADMA accumulation. This effect was significant at 10 U/ml and reached a plateau at 100 U/ml. Similarly, ADMA accumulation was significantly increased by NESP (Figure 1B). Addition of an antibody to EPO (EPO Ab; 10  $\mu$ g/ml) abolished the EPO- or NESP-induced accumulation of ADMA in the same way as the co-incubation with antioxidant PDTC (10  $\mu$ M).

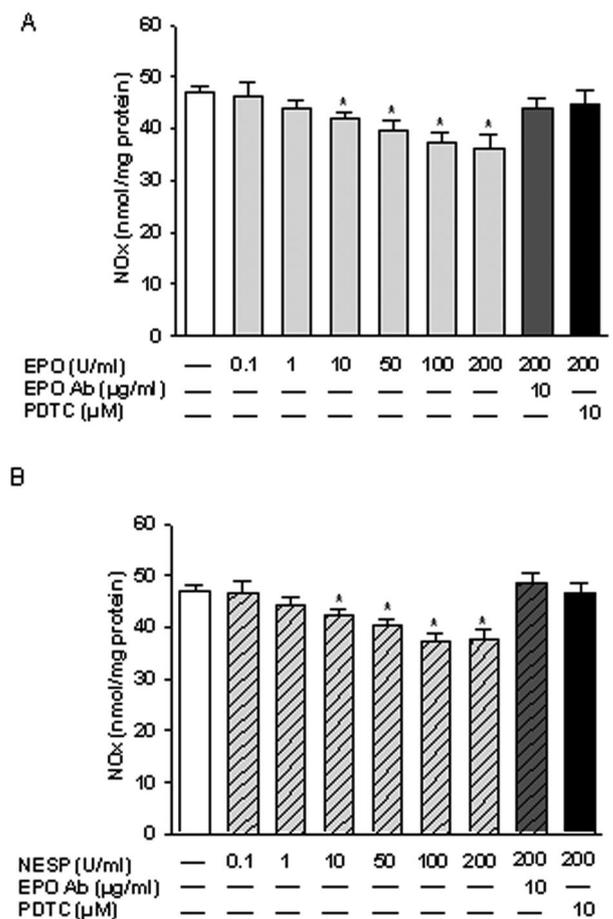
The elevation of ADMA concentration was associated with a reduction in NO formation. EPO reduced NO over 24 h in a dose-dependent manner (Figure 2A). Likewise, addition of NESP decreased the NO synthesis (Figure 2B). Co-incubation with PDTC significantly blocked the effect of EPO or NESP on NO secretion in the same way as the co-incubation with EPO Ab.



**Figure 1.** (A) Effect of epoetin  $\beta$  (EPO) on asymmetric dimethylarginine (ADMA) accumulation in the culture supernatant of endothelial cells. Cells were exposed to control, EPO (0.1, 1, 10, 50, 100, and 200 U/ml), EPO neutralizing antibody (EPO Ab; 10  $\mu$ g/ml with 200 U/ml EPO), or pyrrolidine dithiocarbamate (PDTC; 10  $\mu$ M with 200 U/ml EPO) for 24 h. (B) Effect of darbepoetin  $\alpha$  (NESP) on ADMA accumulation. Endothelial cells were exposed to control, NESP (0.1, 1, 10, 50, 100, and 200 U/ml), EPO Ab (10  $\mu$ g/ml with 200 U/ml NESP), or PDTC (10  $\mu$ M with 200 U/ml NESP) for 24 h. Each point represents the mean  $\pm$  SEM of  $n = 6$  different experiments performed in duplicate. \* $P < 0.05$  versus control.

#### Erythropoietin Decreases DDAH Enzyme Activity by Increasing Extracellular and Intracellular Oxidative Stress

Because ADMA levels were increased by supplemental EPO or NESP, we hypothesized that this accumulation is due to reduce degradation of ADMA by DDAH. Accordingly, we determined DDAH activity by assessing the rate of degradation of exogenous ADMA added to the cell lysates. Concurrent with the significant increase in ADMA formation, DDAH activity significantly decreased in EPO-treated cells in a dose dependent manner (Figure 3A). Comparing different concentrations of EPO added to cell cultures, we observed that DDAH activity reached a minimum value at 100 and 200 U/ml. In an analogue manner, NESP impaired the endothelial DDAH activity (Figure 3B). PDTC enhanced DDAH activity and prevented the impairment of DDAH activity by EPO- or NESP-treated cells in the

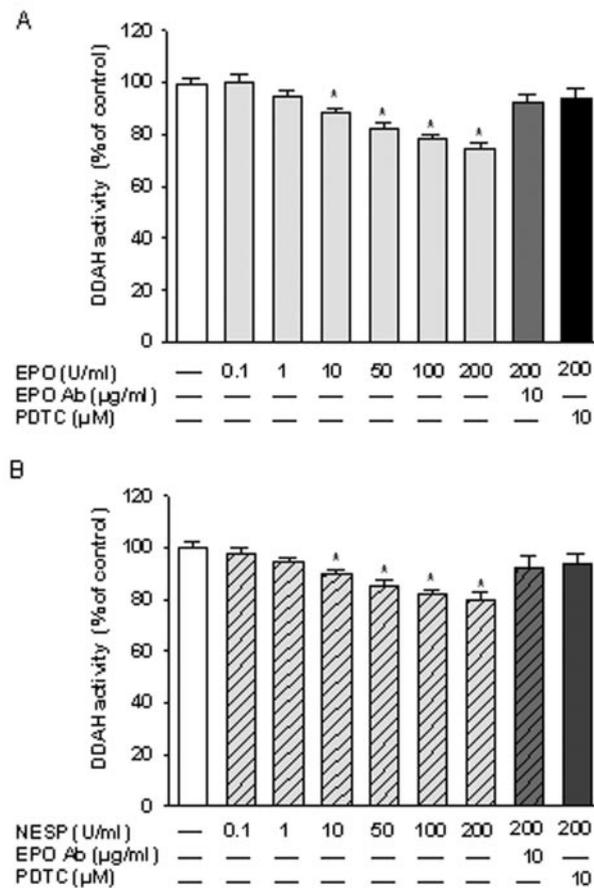


**Figure 2.** (A) Nitric oxide (NO) synthesis by endothelial cells that were stimulated for 24 h in the presence of control, EPO (0.1 to 200 U/ml), EPO Ab (10  $\mu$ g/ml with 200 U/ml EPO), or PDTC (10  $\mu$ M with 200 U/ml EPO). (B) NO synthesis by endothelial cells that were stimulated for 24 h in the presence of control NESP (0.1 to 200 U/ml), EPO Ab (10  $\mu$ g/ml with 200 U/ml NESP), or PDTC (10  $\mu$ M with 200 U/ml NESP). Data are expressed as the mean  $\pm$  SEM of  $n = 6$  different experiments performed in duplicate. \* $P < 0.05$  versus control.

same way as EPO Ab. To attribute whether intracellular oxidative stress is responsible for EPO-impaired DDAH activity, we determined the formation of allantoin in the conditioned culture media and the content of ROS and thiols in endothelial cells.

Incubation for 24 h with EPO dose-dependently increased allantoin levels, a marker of oxygen free radical generation (Figure 4A). The greatest increase in allantoin upon EPO treatment was observed at 100 and 200 U/ml. Alike, allantoin content was significantly increased in NESP-incubated cells (Figure 4B). The stimulatory effect of EPO or NESP on allantoin production was reversed by co-incubating the cells with PDTC or EPO Ab.

The endogenous ROS formation was measured by detection of DHR. The intracellular level of ROS was increased significantly in endothelial cells that were incubated with EPO for 24 h as compared with controls in dose-dependent manner



**Figure 3.** EPO- or NESP-induced accumulation of ADMA was associated with a decrease in endothelial dimethylarginine dimethylaminohydrolase (DDAH) enzyme activity. (A) Endothelial cells were incubated for 24 h in the presence of control, with EPO (0.1 to 200 U/ml), EPO Ab (10  $\mu\text{g/ml}$  with 200 U/ml EPO), or PDTC (10  $\mu\text{M}$  with 200 U/ml EPO). (B) Endothelial cells were incubated for 24 h in the presence of control, with NESP (0.1 to 200 U/ml), EPO Ab (10  $\mu\text{g/ml}$  with 200 U/ml NESP), or PDTC (10  $\mu\text{M}$  with 200 U/ml NESP). DDAH activity in cell lysates from each condition is expressed as percentage of amount of ADMA metabolized by control, which is defined as 100% for every experiment. Each point represents the mean  $\pm$  SEM of  $n = 6$  different experiments performed in duplicate. \* $P < 0.05$  versus control.

(Figure 4C). Induction of ROS production in endothelial cells by EPO reached a plateau at 100 U/ml. EPO Ab reversed the increase in ROS formation. The antioxidant PDTC prevented the effect of EPO on endogenous oxidative stress. Simultaneously, the total intracellular thiol content was measured by detection of CMFDA. In endothelial cells that were incubated with EPO for 24 h, the formation of endogenous thiols was similar to control (data not shown). Taken together, these data suggest an imbalance between oxidant and antioxidant forces in favor of oxidation by EPO-treated cells. Concerning the endogenous ROS and thiol content, NESP showed the same results as EPO-treated cells (Figure 4D).

To provide information about the effect of EPO on ADMA

accumulation in patients who were treated with EPO, we obtained data of three patients who had chronic renal failure and received for the first time EPO. At baseline, ADMA plasma level was  $0.45 \pm 0.014 \mu\text{M}$ , and EPO concentration was  $11.06 \pm 2.68 \text{ mU/ml}$ . After 7 d of EPO (6000 U/wk), plasma ADMA concentration was increased at 16% ( $0.523 \pm 0.022 \mu\text{M}$ ), and EPO concentration reached the value of  $890.80 \pm 49.5 \text{ mU/ml}$ .

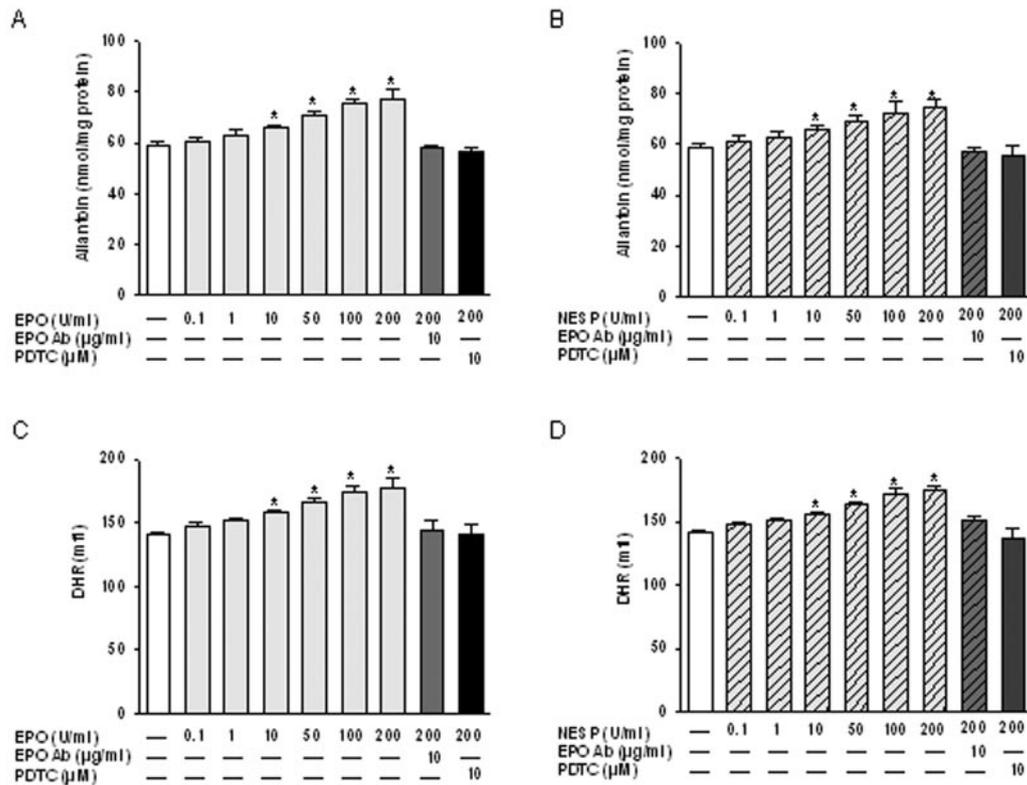
## Discussion

The salient findings of this study are that (1) both EPO and NESP increase ADMA concentration and thereby reduce NO synthesis by endothelial cells in a dose-dependent manner; (2) this effect is associated with a dose-dependent impairment of the DDAH activity, the enzyme that degrades ADMA; and (3) EPO- or NESP-impaired activity of endothelial DDAH is accompanied by a corresponding increase of oxidative stress, which can be prevented by co-incubation with antioxidant PDTC. These data provide the first evidence for a novel mechanism of EPO-induced downregulatory effect on NO production by endothelial cells *in vitro*.

NO generation from the endothelium is important to maintain the vasculature in a relaxed state, inhibit the adhesion of platelets and white cells, and suppress the replication of smooth muscle cells. The reduced bioavailability of NO is a hallmark of vasoconstriction, hypertension, and enhanced platelet activation (18), all of which have been reported as the main side effects of EPO therapy (3–5). In the vascular system, the bioavailability of NO can be impaired by various mechanisms, including decreased NO production by endothelial NOS (eNOS), and/or enhanced NO breakdown as a result of increased oxidative stress. The deactivation of eNOS is often associated with accumulation of its endogenous inhibitor, ADMA (10).

ADMA is synthesized by protein arginine methyltransferase (19), a family of enzymes that methylates L-arginine residues within specific proteins, and is generated from hydrolysis of these proteins. Although a small proportion of ADMA is excreted in the urine, its major route of elimination is the hydrolytic degradation to L-citrulline and dimethylamine by DDAH (20). Two isoforms of DDAH have been identified in every cell type examined: DDAH I is typically found in tissues that express neuronal NOS, whereas DDAH II predominates in tissue that contains eNOS (21). The amount of ADMA within a cell is dependent on the extent of arginine methylation in proteins and the rates of protein turnover, and its accumulation is prevented by its metabolism by DDAH. Pharmacologic inhibition of DDAH causes gradual vasoconstriction of vascular segments, which is reversed by addition L-arginine (12). Given the central role of DDAH in the regulation of ADMA degradation, any pathologic process that decreases DDAH activity would be expected to increase ADMA concentrations, sufficient to diminished NO biosynthesis.

In the experiments presented here, the accumulation of ADMA by EPO- or NESP-treated endothelial cells decreased the elaboration of NO synthesis and was associated with a reduction in DDAH activity. These effects were completely abolished by co-incubation with anti-EPO neutralizing antibody, which suggest



**Figure 4.** EPO- or NESP-impaired DDAH enzyme activity leads to increase intracellular and extracellular oxidative stress. Endothelial cells were incubated for 24 h in the presence of control, EPO or NESP (0.1, 1, 10, 50, 100, and 200 U/ml, respectively), EPO Ab (10  $\mu$ g/ml with 200 U/ml EPO or NESP), or PDTTC (10  $\mu$ M with 200 U/ml EPO or NESP). (A) Effect of EPO on allantoin formation into the conditioned media of endothelial cells. (B) Effect of NESP on allantoin formation. (C) Effect of EPO on reactive oxygen species (ROS) formation measured with dihydrorhodamin 123 using FACS analysis. (D) Effect of NESP on ROS formation. Data are expressed as the mean  $\pm$  SEM of  $n = 6$  different experiments performed in duplicate. \* $P < 0.05$  versus control.

that EPO or NESP were necessary and sufficient for the dysregulation of the DDAH-ADMA-NO system.

The critical role of DDAH activity in regulating NO synthesis *in vivo* was demonstrated by the Cooke group by using a transgenic DDAH mouse (22). This study provides compelling evidence for the importance of DDAH activity and plasma ADMA levels in the regulation of NO synthesis.

The activity of DDAH seems to be related to oxidative stress. A wide range of pathologic stimuli induce endothelial oxidative stress and consequently reduce DDAH activity *in vitro* and *in vivo* (15,23–25). These observations have led to interest in the role of oxidative stress as a mechanism to control the enzyme activity of DDAH. The adverse effect of these stimuli can be reversed *in vitro* by antioxidants, which preserve the activity of DDAH.

Results from this study are consistent with these observations. We found that EPO or NESP increased endothelial oxidative stress measured by intracellular ROS and allantoin production, whereas the total intracellular thiol content was not affected. Allantoin, a marker of oxygen free radical generation, results from further oxidation of uric acid. Allantoin may occur in human tissue exclusively as a result of nonenzymatic reaction with highly ROS (16), because the enzyme involved, uricase, is not expressed in humans. Therefore, elevated levels

of allantoin are considered as a marker of increased oxidative stress. Furthermore, this study indicates that EPO- or NESP-induced intracellular ROS formation is accompanied by a corresponding decrease in the activity of DDAH. This finding is supported by the observation that co-incubation with PDTTC, a thiol antioxidant, reduces ROS formation and consequently the impairment of DDAH activity. This investigation is consistent with the hypothesis that EPO- or NESP-induced oxidative stress impairs DDAH, with a corresponding increase in endothelial ADMA accumulation, which leads to inhibition of NO synthesis.

As reported by many investigators (26–29), EPO interferes with oxidant processes by enhancing ROS formation and oxidant damage to biomolecules in plasma and blood cells in hemodialysis patients. Several pathways for the increased oxidative stress in EPO therapy have been characterized. For example, EPO increases lipid peroxidation in erythrocyte membrane, via reducing hydroxyl radical scavenging activity, and this is positively correlated with serum ferritin level (29). Furthermore, EPO enhances superoxide production by stimulating polymorphonuclear leukocytes *in vivo* and *in vitro* in hemodialysis patients, an effect that could be mediated via activation of the phagocytic isoform of NADPH oxidase (26).

In agreement with our results, exposure to exogenous EPO

results in resistance to the vasodilatory action of NO *in vivo* and *in vitro*, which can contribute to the pathogenesis of EPO-induced hypertension (4). Another potential mechanism proposed for EPO-induced impairment of the NO pathway is that the elevated resting cytosolic calcium concentrations contribute to NO resistance. Binding of EPO to its receptor causes a rise of intracellular calcium levels (30) and thereby inhibits NO production and eNOS protein expression in endothelial cells (7). Recently, it was reported that ATP-stimulated cytosolic calcium increase is followed by an increase in ROS formation in endothelial cells (31).

### Clinical Implications

As reported in the literature, patients with a diagnosis of chronic renal disease received EPO at doses ranging from 50 to 500 U/kg (32–35). Whereas the normal range for plasma EPO is 0.003 to 0.015 U/ml, during a single dose of EPO (100 U/kg) in hemodialysis patients, the plasma levels reached maximal value of 3 U/ml (36,37), approaching the level of EPO or NESP at which we observed an initial *in vitro* response in ADMA accumulation and in NO production. In addition, our observational data concerning patients who had chronic renal disease and received for the first time EPO for 7 d showed that ADMA plasma concentration increased by 16% plasma EPO levels from 0.7 to 0.9 U/ml. Further studies are required to confirm these findings.

### Conclusions

In summary, these data suggest that at concentrations corresponding to plasma levels after EPO therapy in patients with chronic renal failure, EPO and NESP increase endothelial elaboration of ADMA by impairing DDAH activity. Decreased activity of DDAH leads to local accumulation or release of intracellular ADMA and inhibition of NOS. The effect of EPO or NESP to impair DDAH activity is probably mediated by oxidative stress.

ADMA and DDAH are widely distributed in tissue (38) and may provide a mechanism for regulating NO synthesis in physiologic and/or pathologic states. This is a novel mechanism for EPO-induced downregulation of NO synthesis, which may contribute to explain main side effects observed in patients who are treated with EPO.

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