Interleukin-10 inhibits the in vivo and in vitro adverse effects of TNF-α on the endothelium of murine aorta

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Zemse SM, Chiao CW, Hilgers RH, Webb RC. Interleukin-10 inhibits the in vivo and in vitro adverse effects of TNF-α on the endothelium of murine aorta. Am J Physiol Heart Circ Physiol 299: H1160–H1167, 2010. First published July 16, 2010; doi:10.1152/ajpheart.00763.2009.—TNF-α is a proinflammatory cytokine and is an important mediator of maternal endothelial dysfunction leading to preeclampsia. In this study, we tested whether IL-10 protects against TNF-α-induced endothelial dysfunction in murine aorta. In in vivo experiments, aortic rings of C57BL/6 female mice were incubated in Dulbecco’s modified Eagle’s medium in the presence of either vehicle (distilled H2O), TNF-α (4 nmol/l), or recombinant mouse IL-10 (300 ng/ml) or in the presence of both TNF-α and IL-10 for 22 h at 37°C. In in vitro experiments C57BL/6 IL-10 knockout female mice were treated with saline or TNF-α (220 ng/kg -1 day -1) for 14 days. Aortic rings were isolated from in vitro and in vivo experiments and mounted in a wire myograph (Danish Myotech) and stretched to a tension of 5 mN. Endothelium-dependent relaxation was assessed by constructing cumulative-concentration-response curves to acetylcholine (ACh, 0.001–10 μmol/l) during phenylephrine (10 μmol/l)-induced contraction. As a result, overnight exposure of aortic rings to TNF-α resulted in significant blunted maximal relaxing responses (E max) to ACh compared with untreated rings (22 ± 4 vs. 82 ± 3%, respectively). IL-10 knockout mice treated with TNF-α showed significant impairment in ACh responses (E max) compared with C57BL/6 mice treated with TNF-α (51 ± 3 vs. 72 ± 3%, respectively). Western blot analysis showed that endothelial nitric oxide synthase (eNOS) expression was reduced by TNF-α in vitro and in vivo experiments, whereas IL-10 restored the eNOS expression. In conclusion, the anti-inflammatory cytokine IL-10 prevents impairment in endothelium-dependent vasorelaxation caused by TNF-α by protecting eNOS expression.

tumor necrosis factor-α; nuclear factor-κB

tumour necrosis factor-α (TNF-α) is a proinflammatory cytokine responsible for maternal endothelial dysfunction leading to pathological state-like preeclampsia. Several markers have been reported in preeclamptic women, suggesting it as an endothelial cell disorder. Endothelial dysfunction in preeclampsia may disrupt the balance between proinflammatory (TNF-α, IL-1β, IL-2, and IL-6) and anti-inflammatory cytokines, which leads to vascular damage. TNF-α and IL-6 are elevated twofold in preeclampsia. The most common possible mechanism in preeclampsia would be that the factors derived from the placenta produce TNF-α that leads to endothelial disturbances.

TNF-α is a cytokine with antitumoral activity, which has multiple cell functions regulating immune responses. In addition to its proinflammatory actions, there are several reports showing that increased levels of TNF-α are associated with impaired endothelial function (8, 57). This TNF-α-induced endothelial dysfunction is induced by the production of reactive oxygen species (ROS) (14). The pathways activated by TNF-α vary considerably in different conditions and treatments (35, 59). It causes cell death via apoptosis through the activation of nuclear factor-κB (NF-κB). The mechanism of ROS generation by TNF-α is mainly a proapoptotic or cytotoxic effect. These studies have shown that mitochondria play an essential role in the TNF-α-induced ROS generation. An increased production of ROS leads to reduced nitric oxide (NO) bioavailability because of the scavenging of NO (42). NO production is mainly regulated by the endothelial NO synthase (eNOS) enzyme, which is in turn regulated by various other proteins. The pathways activated by TNF-α lead to NF-κB activation and ROS generation, causing an uncoupling of the eNOS enzyme (13, 20, 50). Hence, we hypothesize that TNF-α treatment can cause a downregulation of eNOS expression. NF-κB is activated when it is dissociated from a cytosolic inhibitory protein IκB (24). IκB is then translocated to the nucleus where DNA binding occurs (7, 37, 70). This may lead to a decrease in the eNOS enzyme activity and/or expression, resulting in endothelial dysfunction. Thalidomide inhibits a lipopolysaccharide-induced production of TNF-α (12, 56). Previous studies have also shown that thalidomide blocks NF-κB activation through suppression of IκB kinase activity (40). Studies have reported that IL-10 inhibited the NF-κB activity in purified T lymphocytes (51, 55). Based on these studies, we hypothesized that IL-10 inhibits NF-κB activation and consequently restores the endothelium-dependent relaxation impaired by TNF-α.

In the present study, we investigated whether TNF-α causes an impairment of acetylcholine (ACh)-induced endothelium-dependent relaxation in murine aorta. Our next goal was to determine whether the impairment in endothelium-dependent relaxation caused by TNF-α could be restored by IL-10. To study the protective effects of IL-10, we used isolated aortic rings of wild-type (WT) mice that were treated with IL-10, as well as mice deficient in IL-10. The former studies are referred to as the in vivo effects of IL-10 (see MATERIALS AND METHODS), whereas the latter are referred to as the in vitro effects of IL-10.

MATERIALS AND METHODS

Experimental strategy. For in vitro experiments, aortas from WT mice were divided into four rings of 2 mm length. Each of the aortic rings was incubated in 2 ml DMEM containing either vehicle, TNF-α, recombinant mouse IL-10, or a combination of both TNF-α and IL-10 for 22 h at 37°C. Following incubation, these aortic segments were mounted in a wire myograph to analyze the ACh-induced endothelium-dependent relaxations during contraction with phenylephrine (PE). We hypothesized that IL-10 would restore the impaired ACh-induced relaxation caused by TNF-α by an inhibition of the NF-κB pathway.
For in vivo experiments, we placed osmotic minipumps filled with saline-TNF-α in both WT mice and mice deficient in IL-10. After 14 days, the aortic rings were isolated to perform the functional studies. We hypothesized that TNF-α would cause impairment in ACh-induced endothelium-dependent relaxation when infused in WT mice and IL-10-deficient mice but that this impairment in relaxation would be more in IL-10-deficient mice.

Animals. Experiments were conducted in 12-wk-old female IL-10 knockout (KO) mice and their WT control C57BL/6j mice (JAX mice and services, Bar Harbor, ME). All procedures were approved by the Institutional Animal Care Committee.

Isolation of aortic rings for in vitro studies. Mice were euthanized with pentobarbital sodium (50 mg/kg iv, Abbott, Abbott Park, IL), after which the abdomino-thoracic aorta was excised, placed in ice-cold physiological saline solution (PSS), and cleaned of adhering connective and adipose tissue. Aorta from each mouse was divided into four rings of 2 mm length. Each of the aortic rings was incubated in 2 ml DMEM containing 120 U/ml penicillin and 120 μg/ml streptomycin. The incubation medium contained either vehicle (distilled H2O), TNF-α (4 nmol/l), recombinant mouse IL-10 (300 ng/ml), or a combination of both TNF-α and IL-10 for 22 h at 37°C. Supraphysiological levels of IL-10 (300 ng/ml) were selected for this study, although previous studies suggest that plasma levels of IL-10 are about 2.4 ± 2.1 pg/ml (19). Previous studies have reported that higher plasma levels of IL-10 (505 ± 22.3 ng/ml) had no adverse effects in the subjects (22). Following incubation, the aortic rings were removed and immediately placed in oxygenated PSS with the following ionic composition: (in mmol/l) 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.18 MgSO4·7H2O, 14.9 NaHCO3, 5.6 dextrose, 1.56 CaCl2·2H2O, and 0.026 EDTA. Aortic rings were mounted in a wire myograph (Danish Myotech) filled with 5 ml PSS maintained at 37°C and continuously gassed with a mixture of 95% O2-5% CO2. A resting tension of 5 mN was applied to the aortic rings, and they were allowed to equilibrate for at least 30 min. Endothelium-dependent relaxation was performed on PE-contrasted (10 μmol/l) rings followed by a cumulative concentration response curve to ACh (0.001–100 μmol/l). Endothelium-independent relaxation was tested with sodium nitroprusside (SNP; 0.001–100 μmol/l) during contraction with PE (10 μmol/l).

In vivo experimental protocol and isolation of aortic rings. Mice were anesthetized with ketamine-xylazine, and an osmotic pump containing saline or TNF-α (220 ng·kg⁻¹·day⁻¹) was inserted in the neck of WT mice and IL-10 KO mice as previously described (27). Mice were treated with saline or TNF-α for 14 consecutive days. Mice were divided into four groups: WT mice infused with saline (WT), WT mice infused with TNF-α (WT + TNF), IL-10 KO mice treated with saline (KO), and IL-10 KO mice treated with TNF-α (KO + TNF). Following treatment for 14 days, the mice were euthanized with pentobarbital sodium (50 mg/kg iv, Abbott), after which the abdomino-thoracic aorta was excised. The aorta was either used for performing Western blot analysis or functional studies on the wire myograph.

Blood pressure recordings and treatment. At the end of treatment (14 days), the mice were anesthetized with ketamine-xylazine anesthesia. Following anesthesia, a sterile catheter was inserted into the carotid artery. The incision was closed with a sterile 6-0 Ethicon Ophthalmic Suture. The catheter was secured on the back of the mouse to avoid any biting of the tube by the mouse. All surgeries were conducted under aseptic and sterile conditions to avoid any chances of infection. Once the mouse had recovered from anesthesia, the catheter was connected to a transducer to record mean arterial pressure. Recording measurements of mean arterial pressure were made for 3 to 4 h. Subsequently, the mice were euthanized and the aorta was isolated for functional studies or Western blot analysis.

Western blot analysis. Four aortae were isolated from four mice from the in vivo or in vivo groups as described in In vivo experimental protocol and isolation of aortic rings. Following an overnight incubation or 14 days of treatment, aortae were used for Western blot analysis. Aortic tissue was lysed by lysozyme precipitation assay lysis buffer in the presence of 1 mmol/l PMSF, 1 mmol/l sodium orthovanadate, 1 mmol/l sodium fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for 20 min on ice. Whole tissue lysates were centrifuged at 10,000 g for 20 min at 4°C, and the supernatants were collected. The protein concentrations were determined by the Bio-Rad protein assay. Samples of 50 μg were separated by 10% SDS polyacrylamide gel electrophoresis and transferred by electroblotting onto a nitrocellulose membrane (Hybond; Amersham Biosciences, NJ). For the immunosassay, the membranes were blocked in 5% (wt/vol) nonfat dry milk in 1 X PBS-0.2% Tween 20 for 1 h at 4°C with primary antibodies. Polyclonal rabbit anti-phosphorylated eNOS, anti-eNOS, anti-phosphorylated IkB, anti-IkB, anti-phosphorylated NF-κB, and anti-NF-κB antibody (Cell Signaling) and monoclonal anti-β-actin (Sigma-Aldrich) were used. Immunocomplexes were detected through horseradish peroxidase-conjugated goat antimouse antiserum (Amersham Biosciences), followed by enhanced chemiluminescence reaction (ECL, Pierce Biotechnology).

Drugs. ACh, human recombinant TNF-α, PE, SNP, and thalidomide were purchased from Sigma Chemical (St. Louis, MO). Mouse Recombinant IL-10 was purchased from R&D Laboratories (Minneapolis, MN).

Statistical analysis. Results are presented as means ± SE. Experimental values were calculated relative to the maximal changes from the contraction produced by PE in each segment, which was taken as 100%. The pEC50 values for PE, ACh, and SNP were expressed as −log of the molar concentration to produce 50% of the maximal response. Statistical analysis was performed using two-way analysis of variance to compare the concentration-responses curves between the groups. A standard non-parametric t-test was performed to compare the two groups in Figs. 2, 3, and 4. The analyses were performed using the GraphPad Prism software. Values of P < 0.05 were considered a statistically significant difference.

RESULTS

PE-induced contractions and ACh-induced relaxation in aortic rings treated overnight with recombinant TNF-α: in vitro effects. There was no significant change in the sensitivity (pEC50) to PE in vessels treated overnight with vehicle (7.03 ± 0.14) or recombinant TNF-α (4 nmol/l) (6.89 ± 0.15) or IL-10 (300 ng/ml) (7.02 ± 0.15) or a combined treatment of TNF-α and IL-10 (6.75 ± 0.16). The maximal tension (Emax) in response to PE in vessels treated overnight with recombinant TNF-α (4 nmol/l) (8.00 ± 0.51 mN) was significantly decreased compared with vessels treated with vehicle (10.78 ± 0.57 mN) or IL-10 (300 ng/ml) (11.80 ± 0.67 mN). Emax in response to PE in vessels subjected to combined treatment of TNF-α and IL-10 (10.09 ± 0.69 mN) was similar to other groups (Fig. 1A). The sensitivity to ACh and maximal relaxation with 10 μmol/l ACh after overnight treatment in control-treated aortic rings were 7.16 ± 0.09 and 82 ± 3%, respectively (Fig. 1C). Overnight exposure to TNF-α (4 nmol/l) resulted in an impairment of ACh-induced relaxation (pEC50, 6.25 ± 0.35; Emax, 23 ± 4%; P < 0.05; Fig. 1C). This effect of TNF-α appears to be endothelium dependent since relaxation to the NO donor SNP was similar in aortic rings treated with vehicle or TNF-α (data not shown).

We also examined the effect of exogenously added recombinant IL-10 on ACh-induced relaxations in aortic rings treated with/without TNF-α. Figure 1C shows that IL-10 completely restored relaxation responses to ACh (pEC50, 6.77 ± 0.16; and
$E_{\text{max}}$, 68 ± 4%) in aortic rings treated with TNF-α, whereas those treated with IL-10 alone showed responses similar to control vessels (pEC50, 7.29 ± 0.10; and $E_{\text{max}}$, 79 ± 3%). Relaxation to SNP in aortic rings treated with IL-10 or TNF-α in combination with IL-10 was not different from control vessels (data not shown).

**PE-induced contractions and ACh-induced relaxation in aortic rings from mice treated for 14 days with recombinant TNF-α:** in vivo effects. Sensitivity to PE (pEC50) in the in vivo groups was relatively similar (Fig. 1B; WT, 7.12 ± 0.11; WT + TNF, 7.22 ± 0.11; KO, 7.09 ± 0.13; and KO + TNF, 6.97 ± 0.19). $E_{\text{max}}$ generated by PE in WT mice infused with TNF-α was increased compared with WT mice treated with saline (WT, 7.14 ± 0.30; and WT + TNF, 9.30 ± 0.37 mN). There was no significant difference in $E_{\text{max}}$ between KO mice infused with either saline or TNF-α (KO, 6.23 ± 0.30; and KO + TNF, 5.97 ± 0.19 mN) (Fig. 1B).

In WT mice infused with either saline or TNF-α, pEC50 to ACh and $E_{\text{max}}$ with 10 μmol/l ACh did not significantly differ (7.05 ± 0.14 and 72 ± 3% vs. 7.34 ± 0.08 and 78 ± 2%, respectively; Fig. 1D). However, TNF-α infusion in IL-10 KO mice caused an impairment in the ACh-induced relaxation compared with saline-infused IL-10 KO mice (pEC50, 6.80 ± 0.12; and $E_{\text{max}}$, 51 ± 3% vs. pEC50, 7.12 ± 0.09; and $E_{\text{max}}$, 71 ± 2%; Fig. 1D).

**ACh-induced relaxation in aortic rings treated overnight with recombinant TNF-α:** in vitro effects. We examined the effect of added thalidomide on ACh-induced relaxations in aortic rings treated with/without TNF-α. Figure 2 shows that thalidomide completely restored relaxing responses to ACh (pEC50, 6.6 ± 0.09; and $E_{\text{max}}$, 66 ± 3%) in aortic rings treated with TNF-α, whereas those treated with thalidomide alone showed similar responses to control vessels (pEC50, 6.36 ± 0.14; and $E_{\text{max}}$, 79 ± 6%).

**In vitro treatment with TNF-α decreases endothelium-dependent relaxation by potentiating IκB phosphorylation in murine aorta.** Representative Western blotting images for phosphorylated IκB (pIκB) in samples from aortic rings treated with vehicle, TNF-α, IL-10, a combination of TNF-α and IL-10, or a combination of TNF-α and thalidomide (inhibitor of TNF-α induced NF-κB activation) are shown in Fig. 3A. The protein expression of pIκB quantified via densitometric analysis was significantly increased in aortic rings treated with TNF-α (4 μmol/l) compared with aortic rings treated with vehicle (0.9 ± 0.1 vs. 0.6 ± 0.1; ratio of pIκB to IκB; Fig. 3B). Treatment with IL-10 (300 ng/ml) in the presence of TNF-α normalized/reduced the pIκB protein expression levels to that of aortic rings treated with vehicle alone (0.7 ± 0.1; Fig. 3B). IL-10 treatment alone had no effect on the expression level of pIκB compared with control vessels (0.5 ± 0.1; Fig. 3B).
Thalidomide also reduced the pIκB expression in aortic segments treated with TNF-α (0.6 ± 0.1; Fig. 3, A and B).

In vitro treatment with TNF-α decreases endothelium-dependent relaxation by reducing cytosolic phosphorylated NF-κB expression in murine aorta. Phosphorylated NF-κB (pNF-κB) expression was measured to indicate the amount of NF-κB activated by TNF-α and to elucidate the potential role of IL-10 to inhibit this activation. Representative Western blotting images of pNF-κB expression in samples from aortic rings incubated with vehicle, TNF-α, IL-10, a combination of TNF-α and IL-10, or a combination of TNF-α and thalidomide are shown in Fig. 3C. Protein levels of pNF-κB quantified via densitometric analysis were significantly decreased in aortic rings incubated with TNF-α compared with aortic rings incubated with vehicle (0.3 ± 0.1 vs. 0.8 ± 0.1; Fig. 3D). Incubation with IL-10 in the presence of TNF-α normalized pNF-κB protein expression levels to values observed in aortic rings incubated with vehicle alone (0.8 ± 0.1; Fig. 3D). IL-10 incubation alone decreased the expression level of pNF-κB (0.3 ± 0.1; Fig. 3D). Thalidomide incubation along with TNF-α also restored pNF-κB expression compared with TNF-α-incubated aortic rings (0.7 ± 0.1; Fig. 3, C and D).

eNOS and phosphorylated eNOS expression is reduced by in vitro or in vivo TNF-α treatment. Representative Western blotting images for aortic rings incubated with vehicle, TNF-α, IL-10, or a combination of TNF-α and IL-10 are shown in Fig. 4A. The protein levels of total eNOS quantified via densitometric analysis were significantly decreased in aortic rings incubated with TNF-α compared with aortic rings incubated with vehicle (0.2 ± 0.1 vs. 0.4 ± 0.1; Fig. 4B). An incubation with IL-10 in the presence of TNF-α normalized the eNOS protein expression levels to that of aortic rings incubated with vehicle alone (0.4 ± 0.1; Fig. 4B). IL-10 incubation alone had no effect on the expression level of eNOS compared with control vessels (0.5 ± 0.1; Fig. 4, A and B).

Representative Western blotting images of eNOS expression in aortic rings isolated from four groups, WT, WT + TNF, IL-10 KO, and IL-10 KO + TNF, are shown in Fig. 4C. Since the total eNOS expression levels were similar, we speculated that there might be a change in phosphorylated eNOS. Hence Western blot analysis to address phospho-eNOS expression levels was performed. The protein levels of phosphorylated eNOS quantified via densitometric analysis were significantly decreased in IL-10 KO mice infused with TNF-α (220 ng·kg⁻¹·day⁻¹) compared with IL-10 KO mice infused with the vehicle (0.8 ± 0.1 vs. 0.3 ± 0.1; Fig. 4D). In TNF-α-infused WT mice, no change in phosphorylated eNOS protein expression level was observed compared with saline-infused WT mice (0.6 ± 0.1 vs. 0.5 ± 0.1; Fig. 4D).

DISCUSSION

TNF-α levels are elevated in various vascular diseases like type 2 diabetes, ischemic heart disease, and preeclampsia (34, 52, 53). TNF-α contributes to vascular pathology in these diseases mostly by inducing injury to endothelial cells (61, 68). Injury to endothelial cells (endothelial dysfunction) can be elicited by apoptosis of the cells or by an impairment in the endothelium-dependent relaxation (5, 47). An impairment in the endothelium-dependent relaxation is mainly caused by a decrease in the bioavailability of NO (31, 49). An uncoupling of eNOS enzyme leads to a decrease in NO production and an increase in ROS generation (20). ROS such as superoxide anions can convert NO into peroxynitrite, leading to an impairment in the eNOS-dependent relaxation (6). Studies conducted on human umbilical vein endothelial cells show that TNF-α causes the downregulation of eNOS enzyme (69). On the other hand, an anti-inflammatory cytokine like IL-10 restores eNOS expression impaired by endothelin-1 (72). IL-10 plays an essential role to suppress the production of proinflammatory cytokines like TNF-α (10). Limited studies have been conducted to show the protective effects of IL-10 against vascular damage induced by TNF-α (18, 41, 65a). IL-10-deficient mice develop chronic enterocolitis and show increased levels of TNF-α (60). Based on this correlation between TNF-α and IL-10, we hypothesized that IL-10 restores endothelium-dependent relaxation impaired by TNF-α. We performed in vivo and in vitro experiments to test our hypothesis. Differences in the effects of TNF-α in in vitro and in vivo conditions may be due to various factors. First, in the in vitro assay the phenomenon of IL-10 release from circulating lymphocytes is lacking and supraphysiological concentrations of recombinant IL-10 is delivered exogenously, which is expected to bind to its receptors located on endothelial (11) and smooth muscle cells (45). Second, in vivo studies endogenously produced IL-10 from various immune cells are the only available source to observe its effects on vascular pathology (48). We performed in vivo studies to confirm our findings in in vitro studies to show that the effects of IL-10 are also observed at physiological concentrations. In addition, very few studies have performed vascular studies in mice deficient in IL-10, which were used for in vivo studies (32, 33).

We performed TNF-α infusion using osmotic minipumps for 14 days in KO mice and WT mice. Studies have reported that endothelial dysfunction is associated with various forms of vascular and renal disease, including hypertension, coronary artery disease, chronic heart failure, chronic renal failure, and type 2 diabetes (44). We observed that TNF-α infusion did not...
alter blood pressure (BP) in WT and KO mice, suggesting its association with diseases other than hypertension (data not shown). This is in contrast to our previous study where we found elevated BP in IL-10 KO male mice infused with TNF-α compared with the IL-10 KO male mice infused with saline (BP, 15 ± 4 mmHg) (27). This variation in BP observed in IL-10 KO male and female mice by TNF-α infusion suggests that female mice are protected from a BP rise caused by TNF-α, probably because of enhanced EDHF produced in resistance vessels (23, 38). We found that there was a significant decrease in the PE-induced contraction in vessels incubated with TNF-α compared with vessels incubated with vehicle or IL-10 (Fig. 1A). It could be due to injury to endothelial cells because of apoptosis (15). In vivo studies performed on the KO and WT mice also showed no significant difference in the PE-induced contraction (Fig. 1B). Supporting our studies performed on female virgin mice, a study showed that PE-induced contraction was enhanced only in the pregnant rats infused with TNF-α (10–1,000 pg/ml), whereas in virgin rats the responses were normal (28).

A study showed that TNF-α infusion in vivo depresses the ACh-induced endothelium-dependent relaxation (66). When we analyzed the ACh-induced relaxation in the in vitro-treated aortic rings, there was a significant decrease in the relaxation in TNF-α-treated rings compared with control rings (Fig. 1C). This observation was supported by studies performed on cat carotid arteries, which showed that TNF-α blunted the ACh-induced relaxation. TNF-α contributes to the release of proteins like extracellular-regulated kinase, mitogen-activated protein kinase, and NF-κB translocation that activates the ceramide pathway. This leads to an injury to endothelial cells, thus inhibiting the release of vasodilators like NO or prostacyclin from the endothelium (4, 46, 73). The impaired relaxation was significantly restored when TNF-α was treated in

Fig. 3. Representative Western blot images and corresponding bar graphs showing expression of phosphorylated (p)IkB (A and B) and pNF-κB (C and D) in untreated (control), IL-10-treated (300 ng/ml), TNF-α-treated (4 nmol/l), TNF-α + IL-10-treated, and TNF-α + Thal-treated aortic rings in A and C. Densitometric analysis were performed on untreated (control), IL-10-treated (300 ng/ml), TNF-α-treated (4 nmol/l), TNF-α + IL-10-treated, and TNF-α + Thal-treated aortic rings as shown in B and D. Values are expressed as normalized ratios of the intensities of pIkB to IkB and pNF-κB to NF-κB (n = 4; *P < 0.05) for B and D, respectively.

Fig. 4. A and B: representative Western blot showing expression of the endothelial nitric oxide synthase (eNOS) in untreated (control), IL-10-treated (300 ng/ml), TNF-α-treated (4 nmol/l), and TNF-α + IL-10-treated aortic rings in A. Densitometric analysis were performed on untreated (control), IL-10-treated (300 ng/ml), TNF-α-treated (4 nmol/l), and TNF-α + IL-10-treated aortic rings as shown in B. Values are expressed as normalized ratios of the intensities of eNOS to β-actin (n = 5 to 6; *P < 0.05) for B. C and D: representative Western blot showing expression of the peNOS in 14 days of treatment with saline and TNF-α in WT and IL-10 KO mice. The 4 groups are WT mice treated with saline, WT mice treated with TNF-α, IL-10 KO mice treated with saline, and IL-10 KO mice treated with TNF-α as shown in C. Densitometric analysis were performed on WT, WT + TNF, KO, KO + TNF as shown in D. Values are expressed as normalized ratios of the intensities of peNOS to eNOS (n = 5; *P < 0.05) for D.
combination with IL-10 (Fig. 1C). This suggests that IL-10 inhibits the actions of TNF-α. In vivo studies performed in IL-10 KO mice have shown that IL-10 protects the eNOS-mediated relaxation of carotid arteries by attenuating increases in superoxide production (33). IL-10 inhibits the production of proinflammatory cytokines like TNF-α and IL-6 that stimulate the production of ROS (48). Indeed, in our in vivo studies supported our findings in the in vitro studies. KO mice treated with TNF-α showed significant impairment in ACh-induced relaxation compared with the other three groups (WT, WT + TNF-α, and KO; Fig. 1D). This suggests that IL-10 is essential to protect the vascular endothelium from the effects of proinflammatory cytokines like TNF-α. Lipopolysaccharide is a proinflammatory antigen, and IL-10 protects the endothelium after lipopolysaccharide treatment, supporting our findings (32). We found similar results when TNF-α was infused through osmotic pump in KO mice for 14 days. To detect the proteins involved in the TNF-α-induced impairment in relaxation, we performed Western blot analysis in the samples from the in vitro and in vivo studies. We observed that eNOS expression was reduced in the in vitro samples treated with TNF-α compared with those samples in the control groups. The eNOS expression was restored when TNF-α was treated in combination with IL-10. IL-10 by itself had no effect on eNOS expression (Fig. 4A). In in vivo studies, eNOS expression was not altered in the four groups, whereas phosphorylated eNOS expression was reduced in the KO mice treated with TNF-α compared with the other three groups. This explains the discrepancy we observed in in vitro and in vivo studies. The possible explanation would be that at physiological concentrations, IL-10 would be affecting only the phosphorylated levels of eNOS. Phosphorylated eNOS at Ser1177 shows that eNOS is activated in the three groups except the TNF-α-treated KO mice (17). Thus these results so far show that IL-10 is essential to maintain the eNOS-mediated endothelium-dependent relaxation in aortic rings. In addition, IL-10 restores eNOS expression, which was downregulated by TNF-α.

TNF-α leads to the activation of the NF-κB signal transduction pathway. In unstimulated cells, IκB proteins localize NF-κB dimers in the cytoplasm by masking the nuclear localization sequence of NF-κB. The activation of NF-κB through the receptor activation of TNFR1, IL-1R1, and various Toll-like receptors initiates signal transduction cascades, ultimately leading to the activation of the IκB kinase complex (16, 71). The activation of IκB kinase leads to the phosphorylation of IκB-α and IκB-β at specific serine residues, which targets IκB for ubiquination and subsequent degradation by a proteasome-dependent pathway (2). The degradation of IκB leads to the unmasking of the nuclear localization sequence of NF-κB, allowing nuclear accumulation, DNA binding, and transcriptional activation of target genes (26). Based on this background, we performed protein expression for pIκB and pNF-κB in the in vitro aortic rings. We observed that pIκB expression was increased in TNF-α-treated aortic rings compared with the other groups (Fig. 3A; control, IL-10, TNF-α + IL-10, and TNF-α + thalidomide). The increase in pIκB expression indicates the release of NF-κB and thus its translocation to the nucleus as shown in previous studies (1, 26). Studies have shown that IL-10 inhibited proinflammatory cytokine (IL-1)-induced IκB expression (1). Indeed, in our study, when TNF-α was treated in combination with IL-10 or thalidomide, the phosphorylation of IκB was decreased. Drugs like glimepiride and thalidomide cause an increase in the eNOS activity by the inhibition of TNF-α-induced NF-κB activation (39a, 56). Thalidomide suppresses the NF-κB activation induced by TNF-α and hydrogen peroxide; hence, we used it in vitro studies as a NF-κB inhibitor (40). We observed that thalidomide prevented the decrease in ACh-induced relaxation caused by TNF-α (Fig. 2). This suggests a functional effect of IL-10 similar to thalidomide in restoring endothelium-dependent relaxation, primarily by the inhibition of NF-κB activity. Additionally, pNF-κB was decreased in the TNF-α-treated aortic rings compared with the other three groups (except IL-10; Fig. 3C). The main reason for the decrease in the pNF-κB is due to the nuclear translocation of the phosphorylated protein. Studies conducted on human monocytes have also shown that IL-10 inhibits NF-κB activation (67). Hence, in our studies, we found that when TNF-α was incubated along with IL-10 and thalidomide, the phosphorylation of NF-κB was increased. The decrease in the pNF-κB expression seen in IL-10-treated aortic rings may be due to its inhibitory effects on the phosphorylation of NF-κB, besides its action to inhibit the phosphorylation of IκB. Previous studies have shown that activation of NF-κB and the degradation of IκB lead to endothelial damage, supporting our studies (36). Overall in this study, we emphasize the potential role of IL-10 in preventing endothelial dysfunction induced by proinflammatory cytokines like TNF-α by inhibiting the NF-κB pathway and stimulating eNOS expression. As mentioned previously, elevated levels of TNF-α are observed in various vascular diseases (34, 52, 53). Conditions like preeclampsia show elevated levels of TNF-α, primarily associated with endothelial dysfunction (65). IL-10 on the other hand protects the endothelium against any injury by TNF-α. IL-10 levels are shown to be decreased in preeclamptic patients, whereas higher levels of TNF-α are noted (60a). This would lead us to consider the potential use of IL-10 as a therapeutic drug in the treatment of Crohn’s disease, rheumatoid arthritis, and ischemia-reperfusion injury for thoraco-abdominal aortic aneurysm repair (14a, 39, 62).

In conclusion, our studies demonstrate that TNF-α causes an impairment in the endothelium-dependent relaxation, whereas IL-10 plays an important role to suppress this effect by inhibiting the TNF-α-induced NF-κB activation and restoring the impaired eNOS expression.

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DISCLOSURES

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