Interleukin-10 protects against aging-induced endothelial dysfunction

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Abstract
Carotid and cerebrovascular disease increase markedly with age contributing to stroke and cognitive impairment. Inflammation is a key element of vascular disease. In these studies, we tested the hypothesis that interleukin-10 (IL-10), a potent anti-inflammatory cytokine, protects against aging-induced endothelial dysfunction. Responses of carotid arteries from adult (5 months) and old (22 months) wild-type and IL-10-deficient mice were examined in vitro. Acetylcholine (an endothelium-dependent agonist) produced relaxation in arteries from adult wild-type that was not altered in old mice. In contrast, relaxation to acetylcholine in arteries from old IL-10-deficient mice was reduced by ~50% (P < 0.05). Tempol, a scavenger of superoxide, did not affect responses in adult or old wild-type mice, but restored vasodilation to acetylcholine to normal in old IL-10-deficient mice. Responses of the carotid artery to nitroprusside (an endothelium-independent agonist) were not altered in any group. Vascular expression of IL-6 (a proinflammatory mediator of vascular disease) and components of NADPH oxidase (a major source of superoxide) was increased in old IL-10-deficient mice compared with wild-type (P < 0.05). These findings provide the first evidence that age-related and superoxide-mediated endothelial dysfunction occurs earlier with IL-10 deficiency. Our findings suggest a novel role for IL-10 to protect against age-related increases in expression of IL-6, oxidative stress, and endothelial dysfunction.

Introduction
Aging is the single greatest risk factor for vascular disease (Lakatta and Levy 2003). Starting at approximately the sixth decade of life in humans, the rate of vascular events including stroke increases markedly with age (Rothwell et al. 2005). Carotid artery and cerebrovascular disease greatly increase the risk for ischemic stroke and contribute to cognitive impairment (Lorenz et al. 2007; Wendell et al. 2009; Arntzen and Mathiesen 2011). Despite this impact, relatively little is known regarding the vascular biology of aging as the vast majority of experimental studies have used young or adult models when studying either blood vessels or cells in culture.

Endothelial dysfunction is a key event for both the onset and the progression of vascular disease (Faraci 2011b; Libby et al. 2011). A major component of this dysfunction is the loss of nitric oxide (NO)-mediated signaling that originates normally in endothelial cells and exerts diverse protective effects within the vessel wall and on nearby target cells (Faraci 2011b; Fogel and Pober 2012). Endothelium-dependent and NO-mediated vasodilation decreases with age in both experimental models and in humans (Brown et al. 2006, 2007; Park et al. 2007; Mayhan et al. 2009; Rodriquez-Manas et al. 2009; Faraci 2011a,b; El Assar et al. 2012).

Inflammation plays a major role in vascular disease (Libby et al. 2011; Tabas and Glass 2013). Activation of
inflammatory-related signaling occurs in vascular cells in humans with cardiovascular disease as well as experimental models used to study the impact of cardiovascular risk factors (Donato et al. 2007; Rodriguez-Manas et al. 2009; El Assar et al. 2012; Tabas and Glass 2013). Inflammatory-dependent mechanisms are propagated via intermediate molecules including reactive oxygen species and proinflammatory cytokines (Didion et al. 2009; Johnson et al. 2013; Tabas and Glass 2013). For example, recent studies highlight the importance of interleukin-6 (IL-6) and IL-6 dependent signaling in vascular disease (Rodriguez-Manas et al. 2009; Boekholdt and Stroes 2012; Miwa et al. 2013). While some molecules promote immune-related responses, the anti-inflammatory cytokine IL-10 decreases the magnitude of proinflammatory responses and promotes resolution of inflammation (Ouyang et al. 2011; Tabas and Glass 2013).

Despite evidence that elements of immune-related signaling play a key role in vascular disease, our understanding of mechanisms that regulate these processes is limited, particularly during aging. In this study, we examined the hypothesis that IL-10 normally protects against endothelial dysfunction during aging. To test the hypothesis, we used a mouse model of aging and focused on changes in endothelial function. As noted above, endothelial dysfunction is a fundamental element of vascular disease. Atherosclerosis is an advanced form of vascular disease and endothelial dysfunction is a major contributor to this process. We study carotid arteries because this segment of the vasculature is a common site of development of atherosclerosis and is a site where the clinical consequences of vascular disease are most apparent (Libby et al. 2011). Thus, we are studying mechanisms that underlie early changes in a model of vascular disease. Our findings indicate that genetic deficiency in IL-10 enhanced expression of IL-6 and accelerated endothelial dysfunction suggesting IL-10 normally protects against age-induced endothelial dysfunction.

**Methods**

**Animals**

IL-10-deficient mice (IL-10−/−) used in these studies have been backcrossed more than 12 generations onto the C57BL/6 strain and thus C57BL/6 mice were used as wild-type controls. Mice were fed regular chow and water was available ad libitum. All experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Because we observed no apparent sex-related differences in these experiments, results from both male and female mice were combined. Mice were studied at 5 ± 1 (adult) or 22 ± 1 months of age (old). Body weight was similar in adult wild-type and IL-10-deficient mice: 30.4 ± 1.2 and 27.9 ± 1.5 g, respectively. With aging, body weight was maintained in wild-type mice (31.2 ± 1.4 g), but was decreased somewhat in old IL-10-deficient mice (20.1 ± 0.6 g, P < 0.05).

**Measurements of vascular responses**

Mice were killed with pentobarbital (~100 mg/kg, i.p.). Vessels were removed, cleaned of loose connective tissue, cut into rings and placed into individual organ baths for measurement of isometric tension (contraction and relaxation). To evaluate endothelial function (Faraci et al. 1998; Lamping and Faraci 2001), responses to acetylcholine were measured in carotid arteries following submaximal precontraction (~50–60% of maximum) using U46619 (9,11-dideoxy-11a,9a-epoxy-methanoprostaglandin F2α). Isometric tension contraction and relaxation were measured in isometric tension (Faraci et al. 1998). Nitroprusside is a NO donor and was used to assess endothelium-independent relaxation. Tempol (1 mmol/L), a superoxide scavenger, was used to determine if vascular responses were affected by superoxide. A full dose–response curve to U46619 was obtained at the end of each protocol. We used U46619 because it produces very stable preconstriction in arteries from mice. In addition, this agent is an analogue of thromboxane A2, an important mediator of vascular effects of aggregating platelets.

**Quantitative real-time RT-PCR**

RNA from aorta was prepared using the RNeasy (Qiagen, Germantown, MD) method following extraction with TRIzol reagent (Invitrogen, Carlsbad, CA) (Chu et al. 2002). RNA concentrations were determined using a NanoDrop spectrophotometer, with an OD260/OD280 ratio of greater than 1.9 (indicating very high-quality RNA). Purified RNA (300 ng) was used for reverse transcription reaction (RT) (Chu et al. 2002; Modrick et al. 2012). Identical amounts of RT product were used for real-time PCR with a single well of a 96-well plate containing both TaqMan probes/primer set (Applied Biosystems, Foster City, CA) for genes of interest [with carboxyfluorescein (FAM) fluorophor] and using β-actin (with VIC fluorophor) as a house-keeping gene. Expression levels were normalized to β-actin (4352341E). Relative expression levels were obtained using the ΔΔCt method as described (Chu et al. 2002). Expression of IL-6 (TaqMan primers/probe # Mm00446190_m1), tumor
necrosis factor-α (TNFα, Mm00443258_m1), suppressor of cytokine signaling-3 (SOCS3, Mm.PT.51.7804681), signal transducer and activator of transcription 3 (STAT3, Mm.PT.51.16704475), subunits of NADPH oxidases [Nox2 (Mm00432775_m1), and p22phox (Mm00514478_m1), a major source of reactive oxygen species], as well as endothelial NO synthase (eNOS, Mm00435204_m1) were determined by quantitative real-time RT-PCR using the TaqMan method (Chu et al. 2002). Because angiotensin II contributes to vascular dysfunction with aging (Modrick et al. 2009) and can promote inflammation (Didion et al. 2009), we also measured expression of receptors for angiotensin II [AT1 (Mm01166161_m1) and AT2 (Mm01341373_m1)].

**Drugs**

Acetylcholine, nitroprusside, and tempol were obtained from Sigma (St. Louis, MO) and were dissolved in saline. U46619 (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol with subsequent dilutions made in saline.

**Statistics**

All values are ± SEM. Statistical analysis was performed using repeated measures analysis of variance (ANOVA) followed by the Tukey or Student–Newman–Keuls post hoc test to detect individual differences. A P < 0.05 was considered significant.

**Results**

The endothelium-dependent agonist acetylcholine produced concentration-dependent relaxation of carotid arteries. Compared with wild-type adults, vascular responses to acetylcholine were not significantly altered in old wild-type mice (Fig. 1). Relaxation of the carotid artery to acetylcholine was similar in adult wild-type and adult IL-10-deficient mice. In contrast, responses to acetylcholine were reduced by ~50% in old IL-10-deficient mice (Fig. 1). Relaxation of carotid arteries to nitroprusside was similar in all groups and was not affected by age or genotype (Fig. 1). The latter findings suggest that the dysfunction observed occurred at the level of endothelium and not vascular muscle. Thus, there was no evidence for endothelial dysfunction in carotid arteries from old wild-type mice. In contrast, there was substantial impairment of endothelial function with age in old IL-10-deficient mice.

In wild-type mice, contraction of the carotid artery to the thromboxane agonist U46619 was not affected by age. Responses to U46619 tended to increase in old IL-10-deficient mice, but these differences were not statistically significant (data not shown).

Tempol did not alter responses to acetylcholine in adult or old wild-type mice (Fig. 1). In contrast, relaxation of carotid arteries to acetylcholine in old IL-10-deficient mice was increased by tempol to levels seen in adult and old wild-type (Fig. 1). Regardless of age or genotype, vasodilation to nitroprusside was not affected by tempol (Fig. 1). Similarly, tempol did not affect vasoconstrictor responses to U46619 in old wild-type or old IL-10-deficient mice (data not shown).

To gain additional insight into mechanisms that may contribute to vascular aging and endothelial dysfunction, we measured expression of several genes previously implicated in vascular inflammation and oxidative stress (Fig. 2). There were no significant differences in expression of these genes in adult wild-type versus adult IL-10-deficient mice (Fig. 2). Compared with adult wild-type mice, levels of mRNA for TNFα increased in old wild-type mice compared with adults, but did not change any further in IL-10-deficient animals. Thus, aging alone was sufficient to increase TNFα, and this effect was not modulated by the absence of IL-10. Vascular expression of Nox2 tended to increase with aging in wild-type mice, but this change was not statistically significant. In IL-10-deficient mice, increases in Nox2 with aging were significant. Changes in p22phox (another membrane component of NADPH oxidase) were not detected in old wild-type, but were increased in old IL-10-deficient animals. Expression of IL-6, a cytokine implicated in vascular disease and hypertension, was not altered in old wild-type, but was
inflammation are much less clear. Previous research of endogenous mechanisms that may limit vascular (Libby et al. 2011; Tabas and Glass 2013), the importance of inflammatory-related genes including IL-6 (Brasier 2010). Activation of NF-κB along with components of inflammatory signaling pathways occurs in vascular cells with aging (Donato et al. 2007; El Assar et al. 2012). Through effects on inhibitor of κB (IκB) activity, NF-κB degradation, and DNA-binding activity, IL-10 is a potent inhibitor of NF-κB-mediated effects (Ouyang et al. 2011).

Vascular function and related endpoints appear to be normal in adult IL-10-deficient mice under control conditions. In combination with previous work (Gunnett et al. 1999, 2000, 2002; Didion et al. 2009), the present findings suggest that expression of eNOS, levels of superoxide, vasodilator responses, and vasoconstrictor responses are similar in adult wild-type mice and adult mice lacking IL-10. We and others have found that blood pressure in wild-type mice does not change significantly with aging (Pena Silva et al. 2012; Toth et al. 2013). Similarly, we have shown previously that arterial pressure is similar in adult wild-type and IL-10-deficient mice (Didion et al. 2009). For these reasons, we did not measure blood pressure in the old IL-10-deficient mice. While we assume based on previous work that blood pressure did not change with age, we cannot exclude the possibility that increases in arterial pressure occurred in old IL-10-deficient mice. If arterial pressure increased in these mice, such changes could have contributed to the accelerated endothelial function seen with aging.

Discussion

There are several new findings in this study. Genetic deficiency in IL-10 did not alter vascular function in adult mice, but greatly increased endothelial dysfunction in carotid arteries in old mice. Impairment of endothelium-dependent relaxation in old mice lacking IL-10 was reversed by a scavenger of superoxide suggesting a key role for oxidative stress. Changes in vascular function in old IL-10-deficient mice were not associated with changes in expression of eNOS, but were accompanied by increased expression of IL-6 and components of NADPH oxidase – known mediators of vascular disease. Overall, our findings provide direct evidence that IL-10 plays a major role to suppress age-induced oxidative stress, increases in IL-6 expression, and endothelial dysfunction.

We focused on IL-10 and its potential impact in a model of aging for several reasons. While proinflammatory mechanisms are known to promote vascular disease (Libby et al. 2011; Tabas and Glass 2013), the importance of endogenous mechanisms that may limit vascular inflammation are much less clear. Previous research suggested IL-10 may play a key role in this regard. In nonvascular cells, a major role for IL-10 is to inhibit expression of proinflammatory cytokines including IL-6 (Ouyang et al. 2011). While the impact of IL-10 in both the innate and the adaptive immune system are known, the impact of IL-10 for vascular biology has only begun to emerge. Nuclear factor (NF)-κB (NF-κB) is a transcription factor involved in the regulation of many inflammatory-related genes including IL-6 (Brasier 2010).

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tation, we thought the experimental design used here was appropriate in that it allowed us to test the hypothesis that IL-10 deficiency would augment age-related vascular dysfunction. Alternately, we could have studied mice at >24 months of age. However, the presence of a large effect of age per se on endothelial function in wild-type mice would potentially make it more difficult to detect further dysfunction (and thus test our hypothesis). Based on these issues, the study of mice at ~22 months of age seemed reasonable to us. Our findings in this study indicate that the presence of IL-10 deficiency results in the appearance of prominent endothelial dysfunction at an earlier age.

The major endothelium-derived relaxing factor in the carotid artery is NO (Faraci et al. 1998; Lamping and Faraci 2001). Responses of this vessel to acetylcholine, which are mediated by NO (Faraci et al. 1998; Lamping and Faraci 2001), were greatly impaired in old IL-10-deficient mice, but could be restored to normal by acute administration of a scavenger of superoxide anion. The chemical interaction between NO and superoxide (and resulting inactivation of NO) is a major cause of impaired NO-mediated signaling and endothelial dysfunction in a variety of experimental models and in humans with vascular disease (Faraci 2011b). For example, endothelium-dependent responses in small mesenteric arteries and resistance vessels of the forearm in humans are mediated by NO normally, but impaired with aging (Lauer et al. 2001; Rodriguez-Manas et al. 2009; Angulo et al. 2012; Wray et al. 2012). These reduced responses are improved by scavenging superoxide or treatment with other antioxidants (Rodriguez-Manas et al. 2009; Angulo et al. 2012; Wray et al. 2012). In another study of effects of aging, we provided evidence that scavenging superoxide restores endothelial function (responses to endothelium-dependent agonists) by protecting NO (Modrick et al. 2009). On the basis of this and other previous work (Brown et al. 2006, 2007; Didion et al. 2006; Lund et al. 2009; Modrick et al. 2009), we assume that elevated superoxide was present and was responsible for impairing NO-mediated vasodilation in old IL-10-deficient mice. Thus, the finding in this study that superoxide is a key player in vascular aging in old IL-10-deficient mice is consistent with data in general that oxidative stress may be an important component of vascular aging. Despite the chronic nature of oxidative stress during aging, it is interesting that acute treatment with antioxidants is sufficient to restore endothelial function in both animal models of aging as well as elderly people (Didion et al. 2006; Mayhan et al. 2008; Modrick et al. 2009; Wray et al. 2012). Such results suggest that the vascular abnormalities observed are due to ongoing mechanisms rather than permanent changes in vascular cells.

A prominent source of superoxide in vascular cells is NADPH oxidase (Drummond et al. 2011). The importance of oxidative stress and NADPH oxidase in vascular abnormalities in models of aging as well as in vessels from older humans has been emphasized (Brown et al. 2006, 2007; Park et al. 2007; Mayhan et al. 2008; Rodriguez-Manas et al. 2009; Fleenor et al. 2012). Levels of superoxide and expression of components of NADPH oxidase in the vasculature increase with aging (Brown et al. 2006; Didion et al. 2006; Park et al. 2007; Mayhan et al. 2008; Rodriguez-Manas et al. 2009; Fleenor et al. 2012). Vascular dysfunction with aging is prevented by scavenging superoxide or genetic deletion of the Nox2 component of NADPH oxidase (Brown et al. 2006; Didion et al. 2006; Park et al. 2007; Mayhan et al. 2008; Modrick et al. 2009; Rodriguez-Manas et al. 2009). Our finding that expression of components of NADPH oxidase are increased in old IL-10-deficient mice is further evidence that oxidative stress is a key contributor to vascular abnormalities with aging. We assume that the changes in mRNA seen would be reflected in changes in levels of protein and oxidase activity. A novel aspect of this study is the finding that endogenous IL-10 may normally protect against increased expression of NADPH oxidase during aging. One of the limitations of this study is that we do not have data on local or circulating levels of IL-10. There is not much literature on effects of aging on IL-10 expression, but levels in tissue and plasma have been reported to decrease or not change with aging (Forsey et al. 2003; Saito et al. 2003; Frank et al. 2006).

Diverse mechanisms likely contribute to vascular abnormalities with aging. Although this study implicates an important role for oxidative stress, the findings do not rule out potential contributions by other mechanisms, particularly mechanisms that are driven by, or interact with, reactive oxygen species. Interactions between oxidant- and immune-related mechanisms are well described. Angiotensin II activates NF-κB and increases expression of IL-6 (Schrader et al. 2007; Brasier 2010; Rojas et al. 2010; Johnson et al. 2013), whereas IL-6 promotes oxidative stress via activation of receptors for angiotensin II and NADPH oxidase (Wassmann et al. 2004; Dugan et al. 2009). Endothelial dysfunction in response to angiotensin II requires expression of both IL-6 and the Nox2 component of NADPH oxidase (Schrader et al. 2007; Chrisbollis et al. 2012). While this manuscript was in preparation, a report appeared suggesting that endothelial dysfunction occurs in older IL-10-deficient mice (but not wild-type) via a mechanism that involves cyclooxygenase and vasoconstrictor prostanoids (Sikka et al. 2013). That study focused on aorta and used mice at 9 months of age and older (a precise age was not provided) so detailed comparisons are difficult. However, interactions between
oxidative stress and cyclooxygenase activity are known to exist (Faraci 2011b), so the findings in this study do not rule out potential contributions by other endothelium-dependent mechanisms in older IL-10-deficient mice. Overall, both studies support the concept that IL-10 exerts protective effects that suppress vascular aging.

In summary, oxidative stress appears to be a key component of mechanisms that underlie endothelial dysfunction with aging. This study provides evidence for accelerated oxidative stress and vascular aging in a mouse model that is genetically deficient in IL-10. Endothelial dysfunction in carotid arteries in this model was mediated by superoxide. Data from vascular tissue suggest that changes in expression of NADPH oxidase and IL-6 might contribute to the observed endothelial dysfunction in old IL-10-deficient mice. Collectively, these findings suggest that the balance between expression of IL-10 and mechanisms that promote vascular aging may be a key determinant of the progression of vascular disease. Abnormalities in endothelial cells contribute fundamentally to the onset and worsening of vascular disease. Approaches that target IL-10 or its downstream effectors may have beneficial therapeutic effects to suppress the progression of vascular diseases due to aging.

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Conflict of Interest
None declared.

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